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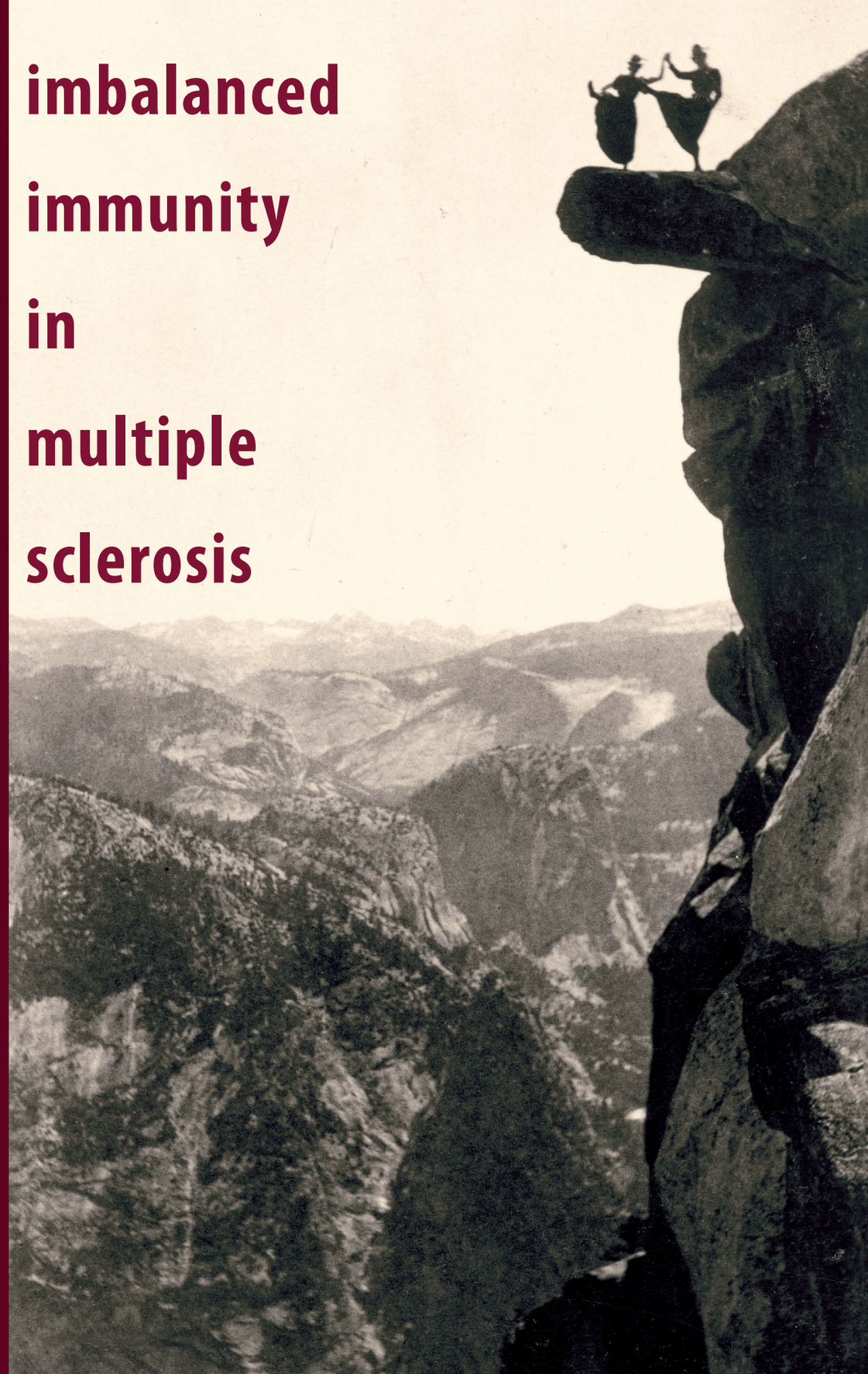

nathalie koning

Imbalanced Immunity in Multiple Sclerosis

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	Dr. B.M.J. Uitdehaag

Faculteit der Geneeskunde

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Contents

Introduction

1

Restoring immune suppression in the multiple sclerosis brain

Scope of the thesis

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Abstract

The inflammatory demyelinating disease multiple sclerosis (MS) is a disabling disease of unknown etiology that often affects adults at young age. The current therapies, mostly aimed to target T cells, can reduce the development of new lesions in the central nervous system (CNS) and partially prevent clinical disease activity. However, no available therapy can cure the disease or even halt its progression. Recent evidence indicates that myelin phagocytosis by infiltrated and disproportionally activated macrophages and microglia is not just a hallmark of MS, but rather a key determinant of lesion development and disease progression. In this review, we will therefore focus on these phagocytic cells, as a primary target for therapeutic approaches to stop the progression of MS. We will first review current therapeutic strategies, which are mostly discussed in literature in terms of their effective inhibition of T cells. However, we argue that many of these treatments also influence the myeloid compartment. The severe side effects of some of these therapies as well as their insufficient effectiveness, necessitates the search for novel therapeutic targets. We postulate that new therapeutic strategies should directly and specifically aim at manipulation of the activation and phagocytic capacity of macrophages and microglia. We will discuss three candidate targets with high potential, namely the complement receptor CR3, CD47-SIRPa interaction as well as CD200-CD200R interaction. Blocking the actions of CR3 could inhibit complement-mediated myelin phagocytosis, as well as the migration of myeloid cells into the CNS. CD47 and CD200 are known to inhibit macrophage/microglia activation through binding to their receptors SIRPa and CD200R, expressed on these cells. Triggering these receptors may thus dampen the inflammatory response. We conclude that the CD200-CD200R interaction is the most specific and hence probably best-suited target to suppress excessive macrophage and microglia activation, and to restore immune suppression in the CNS of patients with MS.

Introduction

Multiple sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system (CNS) affecting over 2.5 million individuals worldwide. MS debuts generally between the age of 20 and 30 and is one of the most disabling neurodegenerative diseases in young adults. The pathological hallmark of MS is white matter demyelination but there are other features that vary between patients, such as axonal and neuronal damage, subpial demyelination, perivascular cuffing of leukocytes, loss of oligodendrocytes as well as anatomical preference of lesions. The heterogeneic neuropathology suggests that it is unlikely that a single pathogenic mechanism underlies MS etiology.[1] Therapies that have been developed so far are aimed at multiple mechanisms such as broad immune suppression or inhibition of migration of immune cells. Some of these therapies reduce the clinical disease activity and the progression of lesion load as determined by magnetic resonance imaging (MRI), but so far no therapy is available that can cure MS or even halt its progression. The search for more effective approaches is therefore warranted.

Evidence is growing that activation of macrophages and microglia is pivotal in the development and expansion of MS lesions.[2-5] It has been observed that clusters of activated microglia are present even before demyelination is evident.[6] Furthermore, infiltrated macrophages and activated microglia are the predominant cell types present in expanding MS lesions[7] and they are actively involved in myelin phagocytosis.[4,8] As long as the cause of MS is unknown, one should therefore particularly target these myeloid cells to dampen inflammation and demyelination in the brain in order to halt progression of MS.

The aim of this review is to focus on the myeloid cell as the common pathological denominator in MS, which will be discussed in the context of current therapeutic strategies. In this respect, we will further discuss the possibilities of the brain's intrinsic macrophage/microglia suppressive mechanisms as a novel therapeutic approach in MS.

Macrophages and microglia: key players in MS

Macrophage populations in the CNS

The main type of immune cells that is found in the CNS belongs to the myeloid lineage and thus to the innate immune system. Myeloid progenitor cells give rise to blood monocytes that in turn can differentiate into macrophages. Macrophages (literally meaning 'big eaters') are phagocytes, providing a first line of defense

against infiltrating pathogens. They express high levels of major histocompatibility complex (MHC) class II molecules and are therefore professional antigen presenting cells as well.[9] After migration into tissues, they can further differentiate into resident macrophages, such as microglia in the CNS. Microglia form the largest population of immune cells in the CNS and are believed to provide a defense against infection and injury.[10] Morphologically they are quiescent ramified cells but despite their inactive phenotype, they continually survey their environment.[11] In contrast to macrophages, however, microglia express low levels of MHC class II, and do not exert substantial antigen presenting properties. Yet upon activation, these cells change their morphology and protein expression profile and are able to phagocytose for example pathogens, apoptotic cell debris, or myelin (as seen in MS).[12]

Other types of CNS macrophages are named after their location, at the interface of the CNS and the peripheral immune system: the perivascular, meningeal and choroid plexus macrophages. The perivascular macrophage is found in the Virchow-Robin space between the endothelial vessel lining and the glia limitans. Although many functions have been attributed to these cells,[13] it mainly involves scavenging of pathogens and other substances derived from the CNS or the circulation,[14] as well as antigen presentation.[15] With increased numbers in the CNS of MS patients and in animals with experimental autoimmune encephalomyelitis (EAE) as well as increased expression of adhesion molecules and chemokines, perivascular macrophages are most likely involved in attracting circulating monocytes/macrophages during MS.[16-18] However, it is unclear whether these cells remain at their location or also invade the brain parenchyma. Meningeal and choroid plexus macrophages possibly remove debris from the cerebrospinal fluid they encounter,[19,20] but it is unclear if and how these macrophages contribute to MS.

The final macrophage population is not present in the healthy CNS, but is frequently found in the CNS of MS patients. These are monocyte-derived macrophages that infiltrate the CNS.[21] In concert with activated microglia, these cells are mainly associated with myelin phagocytosis. The following discussion concerns microglia and macrophages, which here includes perivascular macrophages.

Activation of macrophages and microglia

Depending on the activation signals, macrophages can turn on a classical or alternative activation program.[22,23] Interferon-γ (IFN-γ) or lipopolysaccharide (LPS) stimulation induces classically activated macrophages (also called M1 cells) by signal transduction via mitogen activated protein kinase (MAPK) pathways, including extracellular signal-regulated kinase (ERK)1/2 and p38.[24] M1 cells are typically pro-inflammatory. They express high levels of nitric oxide (NO), cytokines like tumor ne-

crosis factor (TNF), interleukin (IL)-1, -6, -12 and -23 and opsonin receptors, like Fcγ receptors and complement type 3 receptor (CR3), that are necessary for complement- or antibody-mediated phagocytosis. M1 cells can promote T-helper 1 (Th1) responses and thus mediate the elimination of pathogens via inflammation and phagocytosis.[23] In contrast, IL-4, IL-13 or glucocorticoid hormones and their synthetic derivatives like dexamethasone induce alternatively activated macrophages (M2).[22,23] Although the exact intracellular pathways involved in this process are unknown, it is suggested that the anti-inflammatory phenotype of M2 cells results from down-regulation of nuclear factor (NF)-κB and signal transducer and activator of transcription (STAT)1.[23] M2 cells express high amounts of IL-10 and non-opsonin-dependent receptors such as the mannose receptor (MR), and are thought to mediate scavenging of debris, tissue remodeling and repair. M1/M2 polarization likely represents the extremes of a continuum, enclosing many intermediate phenotypes that further fine-tune the different responses.

Although normally quiescent cells, microglia are the first cells to react to any kind of brain insult or injury.[25] They become activated through processes similar to M1 cells, which are initiated by the activation of MAPK pathways.[26] Subsequently, microglia lose their ramifications, upregulate MHC class II molecules and secrete inflammatory substances like NO, IL-1, IL-6 and TNF. Similar to macrophages, activated microglia up-regulate surface receptors like Fcγ receptors and CR3 with which they can participate in antibody and complement mediated phagocytosis.[27]

In patients with active MS lesions, macrophages act mainly pro-inflammatory by secretion of NO, IL-1β and TNF,[28] corresponding with the M1 phenotype. However, it has been reported that foamy macrophages, that result from myelin ingestion, switch their phenotype and start to express markers consistent with M2-like cells.[29,30] This change probably contributes to processes such as gliosis formation by astrocytes. Microglia can also display highly diverse reactions that are associated with both protective and deleterious effects in the CNS.[31] It can therefore be speculated that also microglia activation may be polarized depending on the activation signal, but evidence for this is lacking.

It is still heavily debated what causes the (classical) activation of macrophages and microglia in MS, as is the question whether this activation is followed by, or a consequence of T cell activation, oligodendrocyte apoptosis, or other pathological events. However, macrophages and microglia are considered key players in lesion development and thus in disease progression for a number of reasons. Already in 1952, it was observed that clusters of microglia were present in the MS brain even before cellular infiltrates or demyelination was visible.[32] This was confirmed by many others and has been linked to the earliest stages of lesion development.[6,33,34]

As mentioned above, macrophages and microglia predominate in expanding MS lesions and greatly outnumber lymphocytes.[7] They localize in close proximity to damaged axons[35] and have recently been indicated to phagocytose myelin by a common mechanism mediated by complement and immunoglobulin deposition.[4] Macrophages and microglia furthermore secrete many inflammatory cytokines, reactive oxygen species and chemokines,[26] thereby enhancing inflammation. Interestingly, these data suggest that the disproportional activation of macrophages and microglia is a key event in triggering a cascade of events that ultimately leads to multiple demyelinated areas in the CNS. Therefore, treatments that interfere with the activation of these cells, as well as the process of phagocytosis, would be an excellent approach to halt lesion development and further disease progression.

In fact, it is surprising that none of the currently available or experimental therapies is specifically aimed to halt the actions of macrophages and microglia. Most therapeutic targets were originally developed in EAE and were aimed at limiting T cell activities and migration. Although T cells are required for the induction phase of EAE, their critical role in MS is still elusive.[3] It is therefore not surprising that experimental MS therapeutic approaches aimed at, for example, eliminating $CD4^+$ T cells, seemed beneficial in the treatment of EAE,[36,37] but appeared disappointing in the treatment of MS.[38,39] Notably, despite being induced by T cells, the myeloid cell compartment is essential during the effector phase of EAE.[40-44] We here propose to focus on the role of macrophages, which include perivascular macrophages, and microglia in MS. Interestingly, many experimental therapies, although primarily aimed at T cell activation and function, have effects on demyelinating macrophages and microglia. Hence, we will first highlight these effects of current therapeutic approaches, followed by suggestions for alternatives to more specifically target these cells.

Current therapeutic approaches and their effects on macrophages/microglia

Glucocorticoids

Glucocorticoids (GCs) are known for their broad anti-inflammatory properties.[45] Short courses of high-dose intravenously administered synthetic GCs (i.e. methylprednisolone) are nowadays frequently used to reduce the duration and severity of acute MS relapses.[46] However, the chronic use of GCs is unwanted as it usually causes severe side effects. In addition, despite their frequent use to treat MS re-

lapses, they have no proven beneficial effect on long-term disease progression.[46] The mechanisms by which GCs exert their therapeutic effects are not completely understood, but include altered transcription via GC response elements present in the promoter and enhancer regions of many genes, and interference with other factors such as NF-κB, STAT5 or phosphoinositide 3-kinase (PI3K).[47] GCs are very likely to affect macrophage activities as the glucocorticoid receptor is highly expressed in many macrophage populations, including microglia.[48] The outcome of GC treatment is, amongst others, decreased production and release of pro-inflammatory cytokines like TNF, IL-1β and IL-6 in macrophages and T cells. Also anti-inflammatory cytokines such as IL-10 and transforming growth factor-β (TGF-β) are induced, that subsequently act to suppress these cells.[49] This change in cytokine profile can induce a shift from a Th1 into a Th2 response. However, GCs are also described to induce polarization of macrophages towards an M2 phenotype[23] with similar effects on cytokine production. Thus, direct effects of GCs on macrophages may involve the implicated shift from a TH1 to TH2 response. However, as macrophage polarization by GCs has mostly been studied *in vitro*, this has yet to be proven as an underlying mechanism. Other effects of GCs include preservation of the blood-brain barrier integrity, reduction of perivascular oedema, down-modulation of expression of chemokines, chemokine receptors, adhesion molecules and integrins, that can lead to decreased leukocyte migration.[47] It is however debated whether these are all direct effects of GCs, or whether they result more indirectly for example from the M2/Th2 skewing. Since GCs can freely pass the blood-brain barrier, they can affect cells of the CNS directly, including microglia. Although data on the effects of GCs on microglia are sparse, it has been described that GCs down-regulate TNF produced by activated microglia, prevent phosphorylation of p38 MAPK and decrease the expression of MHC class II molecules.[50,51] Thus, given the fact that macrophages and microglia highly express the glucocorticoid receptor and GCs have profound anti-inflammatory effects on these cells, the effects of GCs on MS relapses may be accounted for by direct effects on macrophage and microglia.

IFN-β

IFN-β is a type I interferon, which is used in the treatment of relapsing-remitting MS and can reduce the frequency of clinical exacerbations.[52] Still, IFN-β does not block lesion development, and can therefore only delay but not halt disease progression. Moreover, a large proportion of treated patients does not respond to the therapy due to the development of autoantibodies against IFN-β.[53] IFN-β has multiple effects on the immune system and due to its non-specific actions, is associated with multiple adverse events such as skin reactions, flu-like symptoms, fatigue and leu-

kopenia.[54] Its therapeutic effects were mostly studied in relation to T cell activation, where it affects many pathways, as shown by microarrays experiments.[55] In addition, it may mediate a shift from a Th1 towards a Th2 response by reducing pro-inflammatory cytokines and induction of anti-inflammatory cytokines.[56-58] Furthermore, the adhesion and migration of T cells, but also monocytes, into the CNS can be diminished because IFN-β inhibits the expression of adhesion molecules on vascular endothelium as well.[59] Despite all efforts, the exact mechanisms by which IFN-β is beneficial in MS are still unresolved. Interesting in the context of this review are the data obtained from studies of endogenous IFN-β, produced by many cell types including fibroblasts, dendritic cells and macrophages. In IFN-β knockout mice, the susceptibility and severity of EAE was significantly increased as compared to their wild type littermates. Whereas in the absence of IFN-β no changes were observed in Th1/Th2 balance or in T cell responses, macrophages and microglia were highly activated.[60] These findings suggest that the protective effect of (endogenous) IFN-β is based on inhibition of macrophages and microglia. This concept was recently corroborated by Prinz *et al.*, who showed aggravation of EAE in mice deficient for type I IFN receptor.[61] In a series of elegant experiments using conditional knockouts for this IFN receptor in different cellular compartments, they showed that there was no effect on the induction and progression of EAE due to absence of the receptor on either nestin-expressing CNS cells (mostly astrocytes) or T cells and B cells. However, upon deletion in myeloid cells, symptoms of EAE were increased and correlated with increased numbers of infiltrated macrophages and activated microglia that further showed enhanced myelin phagocytosing capacity and increased chemokine expression. These reports not only elucidate the action of endogenous IFN-β, but also shed new light on the possible mechanisms by which IFN-β treatment is beneficial in MS patients as IFN-β may thus directly influence macrophage and microglia activities. Although in man several reports have claimed effects on T cells, treatment might thus very well primarily affect the innate immune response.

Glatiramer Acetate

As one of the oldest animal models for a human disease, EAE resulted from 'paralytic' accidents following Pasteur rabies vaccination in human subjects.[62,63] It turned out that these accidents were induced by contaminants of the rabbit CNS tissue that the vaccine was prepared in.[64,65] Thus, traditionally EAE was induced using myelin extracts from different animals like rabbits and guinea pigs, but nowadays the induction of EAE is usually established by inoculating animals with purified myelin components such as myelin basic protein (MBP), or peptide derivatives of these

proteins. In an attempt to further down-scale the complexity of the immunogen, a synthetic derivate of MBP was developed. Copolymer 1 (Cop-1), later known as glatiramer acetate, is a synthetic random polymer of 4 amino acids in a ratio similar to that found in MBP.[66] However, instead of inducing EAE, glatiramer acetate prevented the development and suppressed established EAE induced by spinal cord homogenates or other encephalitogenic peptides.[67] Several clinical trials have shown positive effects of glatiramer acetate on mean relapse rate and proportion of relapse-free patients, but the effect on disability appeared small.[68-70] In the mean time, many studies were conducted to identify the mechanisms of its beneficial effect. Although still not entirely elucidated, two main modes of action were suggested.[66] First, T cells reacting to glatiramer acetate shift from a Th1 towards a Th2 response,[71,72] which is beneficial in MS as shown for most other MS therapeutics. In addition, glatiramer acetate is suggested to have neuroprotective actions, by inducing production of brain derived neurotrophic factor (BDNF) by different T cell lines.[73,74] However, clinical evidence on actions of T cell derived BDNF in MS patients is not available. Second, glatiramer acetate can bind MHC class II of antigen presenting cells such as dendritic cells and macrophages.[75,76] *In vitro* studies show that as the affinity of glatiramer acetate is higher than that of MBP, it can compete with binding of MBP to MHC class II molecules, thereby preventing the activation of MBP-reactive T cells.[77] No data are currently available on effects of glatiramer acetate on other macrophage activities.

Mitoxantrone

Mitoxantrone can be beneficial in relapsing-remitting and secondary progressive MS, but major drawbacks are its adverse effects including an increased risk on developing leukemia, and the restricted cumulative dose a patient may receive during life to avoid cardiac toxicity.[78] As anti-neoplastic agent interfering with DNA synthesis and consequently cell division, mitoxantrone is used for many years to treat malignancies as breast and prostate cancer, and leukemia. Its potential usefulness in the treatment of MS was considered when mitoxantrone appeared to have immune suppressive effects as well. It was shown to block myelin degradation by macrophages[79] and therapeutic treatment of EAE with this compound reduced clinical and histopathological symptoms of the disease.[80-82] In MS, clinical trials have shown that treatment with mitoxantrone reduced gadolinium-enhanced lesions on MRI as well as relapse rate and disability progression.[83-85] On the cellular level, mitoxantrone caused a marked reduction in the proliferation of B cells, as well as a reduced ability of T cells to induce an immune response.[86,87] Remarkably, absence of macrophages entirely abrogated these effects, as shown in EAE,[87] indi-

cating that it is the macrophage that is primarily affected by mitoxantrone which subsequently has suppressive influences on T and B cell activities. These results implicate that mitoxantrone may exert its therapeutic effects in part by affecting macrophages.

Anti-VLA-4

During any inflammatory disease, leukocyte infiltration into the inflamed tissue starts with the process of tethering, rolling and firm adhesion to the endothelial cells of the microvasculature.[88] In MS, immune cells transmigrate through the endothelium and can enter the brain parenchyma if they are able to pass the glia limitans, which is the final component forming the blood-brain barrier.[89] Many molecules are involved in this cascade of events that have consequently been proposed as targets to interfere with leukocyte invasion in the MS brain.

One of the most encouraging therapies currently available is a monoclonal antibody against the 'Very Late Antigen-4' (VLA-4). The integrin VLA-4, a heterodimeric molecule composed of an α4 and β1 chain, is expressed on most leukocytes, and binds to the vascular cell adhesion molecule (VCAM)-1 on vascular endothelial cells. This interaction is a key event in the arrest of leukocytes on the endothelium. The humanized monoclonal antibody against VLA-4, natalizumab, successfully reduced relapse rates and disease progression in MS patients.[90] Simultaneously however, several reports claimed adverse effects of VLA-4 antagonists. Natalizumab was temporarily withdrawn from the market when three patients developed progressive multifocal leukoencephalopathy (PML), an often fatal disease caused by the JC virus, usually in immuno-compromised patients. Two of these patients were also treated with IFN-β and the third patient received additional therapy for Crohn's disease.[91-93] It is still unclear if the intense immune suppression allows the virus to replicate and spread, or whether the treatment directly influences virus activity.[94] After a review of safety information and no further cases of PML, the drug was returned to the market in 2006. Recently, two further incidents of PML during treatment for MS were reported (http://www.fda.gov/cder/drug/InfoSheets/HCP/natalizumab2008HCP.htm). Both patients were on monotherapy for more than one year. Despite these new cases, the drug will stay available for the treatment of MS. Although anti-VLA-4 is considered one of the most successful therapies in MS so far, and having less severe side effects than glucocorticoids or IFN-β, also this therapy is not able to stop progression of MS.

In contrast to broad immunosuppressants as glucocorticoids, IFN-β and mitoxantrone, natalizumab was specifically designed to limit T cell migration. The reason for this was that in the early 1990s, the first evidence was provided that

interaction between VLA-4 and VCAM-1 was a crucial migratory mechanism in MS, as VCAM-1 was highly expressed in MS and EAE,[95,96] and antibodies blocking this interaction prevented leukocyte adhesion and infiltration into the CNS and inhibited the induction of EAE.[97-100] Recently it was shown that circulating monocytes and B cells express significantly higher levels of VLA-4 compared to T cells.[101] It is therefore not surprising that natalizumab significantly reduced not only the number of T cells, but also of macrophages and B cells in the cerebrospinal fluid in treated patients.[102] So despite being specifically aimed at T cells, natalizumab may additionally influence migration of other cell types, including that of macrophages.

Macrophage/microglia: targeted immune suppression

CR3

Apart from monocyte/macrophage infiltration in the CNS, the activation and subsequent myelin phagocytosis by macrophages and microglia are crucial in MS lesion development as mentioned earlier. Molecules specifically involved in these processes would be interesting targets for MS therapy. An appropriate candidate is the complement receptor 3 (CR3), which is constitutively expressed on both macrophages and microglia.[103] Like VLA-4, CR3 is an integrin, also known as Mac-1. CR3 consists of CD11b (αM) and CD18 (β2) chains, and binds the intercellular adhesion molecule ICAM-1 on endothelial cells and the C3b component of complement. Through these interactions CR3 is involved in monocyte/macrophage adhesion, transmigration and phagocytosis predominantly of targets opsonized by complement.[104-106] CR3 expression levels are increased on activated microglia, as seen in the facial nerve transsection model.[107] Blocking CR3 appears highly efficient in EAE as antibodies against CR3 inhibited both leukocyte adhesion via interaction with ICAM-1 as well as phagocytosing activity of macrophages, thereby preventing the disease from progressing.[108,109] Furthermore, activation of microglia could also be blocked by CR3 inhibition, which was also correlated with suppression of EAE.[110] These data strongly suggest that the inhibition of this molecule could be attractive as MS therapeutic (Fig. 1), but so far this target has not been explored for a clinical trial.

Intrinsic immune suppressive mechanisms

The CNS comprises several intrinsic mechanisms that tightly regulate the activities of microglia. This is important, since an uncontrolled inflammatory reaction could be detrimental to a tissue that is notoriously known for its poor regenerating capacity. Normally, quiescent microglia remain capable of inspecting and guarding

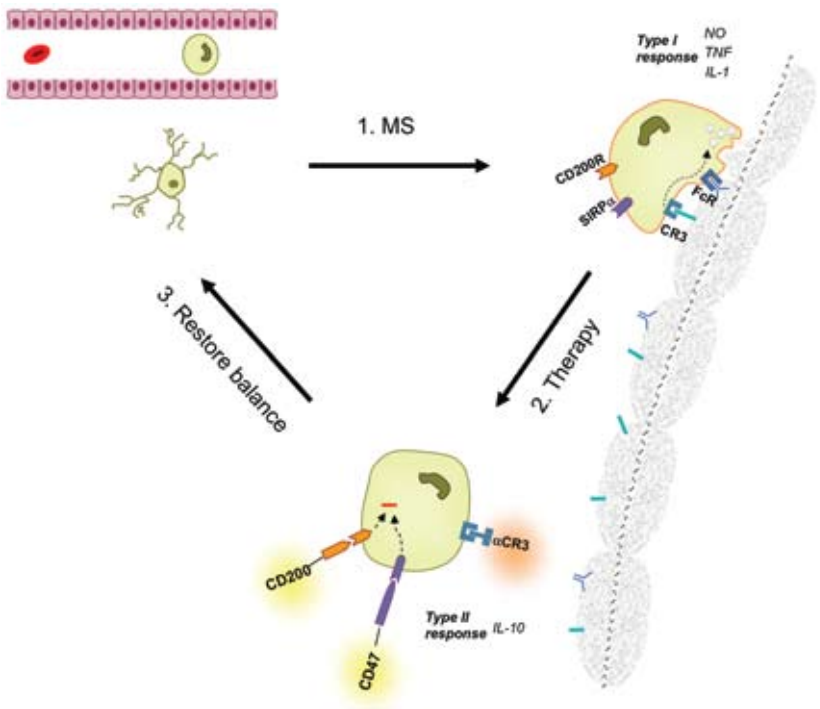

Fig. 1 The as yet unexplored therapeutic targets on myeloid cells in MS. Infiltrated macrophages and microglia are highly activated in MS (step 1). These classically activated cells secrete NO and type I cytokines such as IL-1β and TNF. Via high expression of the complement receptor CR3, these cells mediate complement-activated myelin phagocytosis. In addition, in MS lesions the ligands for the inhibitory receptors SIRPα and CD200R are not sufficiently present to suppress cellular activation. Thus, by blocking CR3 and triggering of SIRPα and CD200R, phagocytosis and pro-inflammatory activities of the cells are inhibited and the pathological phenotype is shifted towards alternatively activated macrophages (step 2). These interventions drive these cells to mediate type II immune responses (e.g. IL-10 production) that involve tissue repair and dampening of inflammation. Eventually, the imbalance in immune homeostasis in the brain will be restored by enhancing the brain's intrinsic immune suppressing potential (step 3).

their environment, as shown by Nimmerjahn *et al.*[11] Only when a certain threshold is exceeded, they become activated and typically show retraction of ramifications, rounding off of cells and up-regulation of a number of markers like CD45 and MHC class II. These cells will become motile, phagocytic and might even display local proliferation. A proper balance in immune activating and immune inhibitory signals thus regulates their activation. We and others have postulated that excessive microglia/macrophage activation in a pathological setting is due to imbalanced control, reflected by impaired immune activation, immune inhibition, or both, and could lead to disease progression as seen in MS.[111,112] This view subsequently implies that correcting the equilibrium by supplying extra inhibitory signals specific

for these cells may dampen the pathological inflammatory response and restore the immune suppressed environment of the CNS (Fig. 1). Eligible specific candidate therapeutic targets to diminish inflammation in MS via these routes are the immune inhibitory molecules CD47 and CD200, and their receptors signal regulatory protein (SIRP)α and CD200R.

CD47-SIRPα

CD47, also known as integrin-associated protein, is a membrane glycoprotein and belongs to the immunoglobulin superfamily (IgSF). It is broadly expressed in different cell types and abundantly present in the CNS, amongst others on neurons. Its receptor, SIRPα, is expressed on neurons and on myeloid cells. The effects of SIRPα-signaling in neurons is unknown, but CD47 ligation to myeloid SIRPα has been shown to mediate cellular inhibition in these cells, as phosphorylation of immunoreceptor tyrosine-based inhibitory motifs in its cytoplasmic tail leads to reduced MAPK activation.[113] CD47 can not only reduce macrophage activation, it can also prevent phagocytosis as erythrocytes derived from $CD47^{-/-}$ mice were instantly cleared by macrophages when transferred into wild type mice.[114,115] Furthermore, blocking CD47 also inhibits the migration of monocytes across brain endothelial cells.[116,117]

As excessive macrophage and microglia activation occurs in the CNS of MS patients, we recently hypothesized that in MS lesions, the immune suppressive signals such as from CD47 were reduced. Therefore we studied the expression patterns of CD47 in relation to several other (inflammatory) molecules in three different sub-areas from chronic active and inactive MS lesions.[111] Indeed, the expression of CD47, but not of SIRPα, was reduced in MS lesions. In the rim of chronic active lesions this coincided with increased complement levels, a profile that is known to promote phagocytosis. Interestingly, this profile was also found in the area surrounding chronic active lesions, where lesion expansion is likely to occur. In contrast, this expression profile was absent in the area surrounding inactive lesions, where lesion expansion has halted. These results indicate that in chronic active lesions, this pro-inflammatory and pro-phagocytic environment may facilitate and contribute to lesion development, and that SIRPα signaling can contribute to curtailing lesion expansion.

Collectively, these data suggest that treating MS patients with a SIRPα agonist could reduce monocyte migration into the CNS and decrease the activation and phagocytic capacity of macrophages (Fig. 1). A few obstacles however include that both CD47 and SIRPα can induce intracellular signaling pathways, which are not entirely understood. Hence a SIRPα agonist might turn out to also act as CD47 antagonist. As $CD47^{-/-}$ animals have no clear phenotype,[118] CD47 is a potential therapeutic target, however, in need of further experimental investigation.

CD200-CD200R

CD200 is also a membrane glycoprotein belonging to the IgSF. Its expression is extremely high in the CNS, where amongst others, neurons are positive.[111,119,120] Having a short cytoplasmic tail with no known signaling motifs, it is unlikely that CD200 itself exerts functions in the cell on which it is expressed. Its receptor, CD200R, is homologous to CD200, but its expression is confined mainly to myeloid cells like macrophages and microglia,[119] and to a subset of B and T cells.[121,122] CD200R has an extended cytoplasmic tail, which contains three tyrosine residues, one of which forms part of an NPXY motif. Upon binding to its ligand, the tyrosine residues become phosphorylated, and adaptor proteins downstream of tyrosine kinase (DOK)1 and 2 are recruited. This ultimately leads to inhibition of the MAPK p38, ERK and c-Jun N-terminal kinase (JNK), the common pathways involved in classical activation of macrophages and microglia. This in turn results in inhibition of release of several cytokines like TNF, IL-5 and IL-6.[123,124] Thus, through CD200-CD200R interaction, the activity of macrophages and microglia can be down-regulated. The strikingly high expression of CD200 in the CNS is therefore thought to be a mechanism of constitutive immune suppression in this sensitive organ. Indeed, blocking CD200-CD200R interaction in $CD200^{-/-}$ mice, leads to spontaneous activation and increased proliferation of microglia and macrophages, demonstrated by upregulation of CD45, which is a hallmark of microglial activation, and expression of inducible nitric oxide synthase (iNOS).[125] Induction of EAE and experimental autoimmune uveoretinitis (EAU) showed enhanced macrophage infiltration and a more rapid and severe disease course compared to wild type mice.[125-127] Also in rats, a blocking antibody against CD200R aggravated EAE which was reflected by enhanced infiltration of T cells and activated macrophages.[121,128] In the latter study, it was further shown that interruption of CD200-CD200R interaction significantly increased neuronal damage in macrophage-neuronal co-cultures. Taken together, these data implicate that CD200 is a potent immune suppressor and that reduced inhibitory input from CD200 causes a disturbed equilibrium, which subsequently results in cellular activation and tissue damage. Recent data from our lab confirmed this in MS. Besides a decreased expression of CD47, we also found decreased expression levels of CD200, but not CD200R.[111] Immune inhibition may thus be hampered in MS and could facilitate the activated state of macrophages and microglia with their demyelinating activity as a consequence.

A crucial finding in animal studies shows that increased CD200R signaling can be beneficial. In mice that have inherently elevated levels of CD200, EAE is ameliorated and is accompanied by enhanced neuroprotection.[129] As expected, in these animals fewer activated macrophages were present in the CNS and less evidence

was found for disrupted myelin sheets and axonal damage. Interestingly, a study recently conducted in our lab revealed that CD200R expression is increased on alternatively activated macrophages compared to classically activated macrophages (manuscript submitted). CD200R is very likely to mediate several actions of M2 cells such as the reduced secretion of inflammatory cytokines.[124] In addition, CD200R expression on T cells also appeared to be restricted to Th2 cells,[130] and finally, it may down-modulate the Th1 response as shown in a study where tumor cells overexpress CD200.[131] Conclusively, these results imply that using a CD200R agonistic agent in MS patients could suppress macrophage/microglia activation, restore the intrinsic immune suppressed environment of the CNS and could thereby restrain disease activity (Fig. 1). Furthermore, it may shift Th1 to Th2 responses, a paradigm many broad immune suppressants rely on. Although by suppressing macrophages caution should be taken with respect to the development of opportunistic infections, targeting CD200R would be more specific than most other immune suppressants because its expression pattern is restricted and its effects have been well studied, which would consequently decrease the probability of unexpected adverse events.

Conclusion

Inhibition of blood-brain barrier disruption, migration/infiltration or myelin phagocytosis by myeloid cells all seem plausible mechanisms to limit CNS inflammation in MS. Current therapies, although not directly tailored to do so, do have effects on macrophages and microglia. However, specifically targeting the activation of these cells would likely increase the effectiveness of the treatment, especially when using the CNS' intrinsic immune suppressive systems. Direct suppression of macrophages and microglia as MS therapeutic is an unexplored field. As of yet, CD200 seems the most suitable candidate to restore the immune suppressive status of the CNS in MS. Moreover, since it is not likely that CD200 and CD200R have other binding partners, the chances of multiple adverse effects as seen with the broad immune suppressants might be considerably lower. Interestingly, although beyond the scope of this review, these potential therapeutic targets may be equally well appropriate in preventing damage caused by activated microglia in other neurodegenerative disorders like Alzheimer's disease[132] and Parkinson's disease.

Scope of the thesis

As discussed above, activated macrophages and microglia are thought to be crucial in the development of MS lesions. How the activation of these cells is regulated in MS is not completely understood, while this knowledge could have a significant impact on treatment strategies. The inappropriate activation of macrophages and microglia in MS reflects an imbalanced immune regulation in the brain, which can be due to enhanced activating signals, decreased inhibition or a combination of both. Most of the studies addressing this issue in MS focused on activating signals. However, in the immune-privileged CNS, there are some powerful systems that continuously suppress local immune activities that have hardly been studied. Previous work showed that absence of only one of these inhibitory molecules, CD200, was sufficient to cause spontaneous activation of microglia in mice and to accelerate the development of EAE, the animal model for MS.[125] These results demonstrate the importance of constitutive immune inhibition in the brain. In the present thesis we hypothesize that immune suppressive signals in MS lesions are diminished, thereby contributing to imbalanced immunity. The consequence is facilitated activation of macrophages and microglia that in turn is associated with lesion formation and disease progression. The aim of the studies described in this thesis was therefore to study the expression patterns of immune suppressive systems in the brains of MS patients as compared to brains of healthy donors, as well as the regulation of these systems *in vitro*. These studies mainly focused on immune suppression via CD200-CD200R, but also CD47-SIRPα and, in the periphery, GITR-GITRL interaction, are regarded as potent immune regulatory mechanisms.

In the first study, described in **chapter 2**, we used laser dissection microscopy to isolate different areas of chronic active and inactive MS lesions to discriminate between lesion compartments that reflect different inflammatory stages in lesion development. Within different lesion areas, we analyzed the expression patterns of immune inhibitory molecules CD200-CD200R and CD47-SIRPα in relation to inflammatory mediators, and other molecules thought to be involved in lesion development. To further understand the interactions of CD200 and CD200R in the CNS, we investigated the anatomical and cellular localization of these molecules in the human CNS, as shown in **chapter 3**. Next, it was important to know if and how the expression of CD200 and CD200R can be influenced, which is important in view of the development of therapeutic approaches. In **chapter 4** we have studied the effects of different pro- and anti-inflammatory stimuli on CD200R expression on human monocyte-derived macrophages. However, not only macrophages, but also activated microglia play a predominant role in MS lesion formation. **Chapter**

5 describes a unique procedure to isolate and culture human post-mortem microglia. In a pilot study, we report on the expression and regulation of CD200R on these cells and compared them to other CNS-associated macrophages. In addition, we addressed the regulation of CD200 on a human neuroblastoma cell line. In **chapter 6** we focused on a peripheral mechanism of immune suppression by way of regulatory T cells. We show the effects of constitutive GITR-GITRL interaction and its functional consequences in EAE, the animal model for MS.

The main conclusions of the present thesis are that constitutive expression of immune suppressive molecules such as CD200 and CD200R is essential in maintaining an immune suppressed environment in the CNS. Diminished expression of these molecules in MS lesions can contribute to the imbalanced immunity in MS, reflected by hyperactive macrophages and microglia. Our data presented here suggest that targeting CD200-CD200R interaction would not only result in decreased immune activation, but may also cause the immune reaction to be shifted towards a more beneficial anti-inflammatory response, thereby affecting inflammation, demyelination and axonal damage, the hallmarks of MS.

Immune suppression in MS

2

Down-regulation of macrophage inhibitory molecules in multiple sclerosis lesions

Nathalie Koning, Lars Bö, Robert M. Hoek, Inge Huitinga

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Abstract

Inflammatory and demyelinating activity of activated resident macrophages (microglia) and recruited blood-borne macrophages are considered crucial in multiple sclerosis (MS) lesion development. The membrane glycoproteins CD200 and CD47, highly expressed on neurons, are mediators of macrophage inhibition via their receptors CD200R and SIRPa, respectively, on myeloid cells. We determined the expression pattern of immune inhibitory molecules in relation to genes involved in macrophage activation and MS lesion pathology. Laser dissection microscopy was combined with real time-PCR to quantitatively study these gene expression patterns in specific sub-areas (i.e. rim, centre and normal appearing white matter) of chronic active and inactive MS lesions. Hallmarks of MS pathology were confirmed by mRNA expression patterns of GFAP, NF, MBP, growth factors, chemokines and receptors, and macrophage activation markers, although expression of osteopontin and αB-crystallin was decreased. CD200 and CD47 were down-regulated in the centre of chronic active and inactive MS lesions. CD47 expression was also decreased in the rim of chronic active lesions, where complement expression was increased. This expression profile was also found in normal appearing white matter surrounding these lesions, but not surrounding inactive lesions. Expression of CD200R and SIRPa was not altered. These data suggest that diminished immune inhibition via decreased CD200 and CD47 expression contributes to a disturbed equilibrium in macrophage and microglia activation in MS lesions. Furthermore, this may result in a pro-inflammatory predisposition in the area surrounding chronic active lesions, thereby contributing to axonal injury, demyelination and possible lesion expansion.

Introduction

Multiple Sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system (CNS) with unknown etiology. Inflammation is a possible primary feature of demyelination[133] and has been shown to be correlated with axonal damage that may reflect the progressive decline in MS.[134] Inflammatory macrophages are likely to play a crucial role in lesion development, also demonstrated in animal models of MS.[42,43,108] The extent and distribution of macrophage infiltration can be used to categorize the inflammatory activity of MS lesions.[34,135] The highest level of inflammation is found in the hypercellular active lesion, with perivascular infiltration and myelin laden macrophages. This is followed by a chronic active stage, defined as having a hypocellular and demyelinated gliotic centre, surrounded by a hypercellular rim containing foamy macrophages. When the inflammation resolves, few macrophages remain present throughout the lesion which is now considered inactive. At present, it is not understood which factors promote inflammation or the transition from chronic active into inactive lesions. Understanding how the inflammatory and possible demyelinating activity of macrophages and microglia increases during lesion formation and why progression at some point stops would help to comprehend MS pathology and will possibly contribute to development of new therapeutic strategies.

Macrophage behavior is tightly regulated by the integration of activating and inhibitory signals. The membrane glycoprotein CD200 is such an inhibitory ligand which is broadly expressed and abundantly present in the CNS and provides an inhibitory signal to myeloid cells, including macrophages and microglia, via the CD200 receptor (CD200R).[121,125,136] Inhibition through CD200R signalling occurs via phosphorylation of its cytoplasmic NPXY motif that recruits DOK1, which subsequently inhibits the Ras/MAPK pathways.[123,124] Mice lacking CD200 have an expanded and activated myeloid compartment and show a significantly accelerated development of experimental autoimmune encephalomyelitis (EAE), an animal model for MS.[125] A second immune inhibitory receptor is signal-regulatory protein α (SIRPα or SHPS-1).[137,138] SIRPα is expressed on myeloid cells and neurons and is triggered by its ligand CD47, which, like CD200, is also highly expressed in the CNS.[139-141] CD47-SIRPα interaction causes inhibition of macrophage activity via activation of immunoreceptor tyrosine-based inhibitory motifs (ITIMs), present in the cytoplasmic part of SIRPα.[113,142,143] Furthermore, this interaction has been shown to be important in the inhibition of phagocytosis by macrophages.[144]

The abundant expression of CD200 and CD47 suggests an important role for immune inhibitory molecules in the CNS, a site that typified as being immunologically

privileged. However, it is unknown whether expression of inhibitory molecules in the CNS of MS patients is altered in a way that it might contribute to an environment with an inflammatory predisposition. Since the activation of macrophages and microglia is pivotal in MS, research has so far focused on factors stimulating this activation. However, the number of studies on immune inhibitory molecules, that may in principle be equally important in MS lesions, is limited.

In the present study we analyzed gene expression profiles of CD200, CD200R, CD47, SIRPα, and other macrophage regulatory molecules in relation to a set of genes thought to be involved in the development of MS lesions. We show that in and around lesions in MS brains, immune suppression may be hampered relative to non-MS controls, since expression of CD200 and CD47 in MS lesions is decreased.

Methods

Subjects

Snap-frozen tissue specimens of 19 MS patients containing white matter lesions and white matter tissue of 10 controls obtained at autopsy were provided by the Netherlands Brain Bank, Netherlands Institute for Neuroscience, Amsterdam. Permission was obtained for brain autopsy and the use of the tissue and clinical information for research purposes. All MS patients were clinically diagnosed by a neurologist (Prof. C.H. Polman, VUMC, Amsterdam), and this diagnosis was confirmed post-mortem by a neuropathologist. Exclusion criteria for MS patients and controls were treatment with immune suppressive agents in the last three months before death, death from bacterial sepsis and having neurological disorders other than MS. At the time of death, fifteen MS patients had a secondary progressive form of the disease. One patient had a primary progressive disease course, one had a relapsing-remitting course and for two patients, the disease course could not be determined. Control subjects were age and gender matched. Mean post-mortem delay (PMD) was 8.28 h (range 4.15 – 17 h) for MS patients and 8.43 h (range 4.50 – 22.15) for control subjects. Mean age of MS patients was 59.9 years and 63.4 years for control subjects. Differences were not significant. Detailed patient characteristics are shown in Table 1.

Immunohistochemistry

Lesion inflammatory activity was characterized as described previously,[34,135] in cryostat sections (8 µm) stained for proteolipid protein (PLP) (Serotec, Oxford, UK) and HLA-DP, -DQ, -DR (DakoCytomation, Glostrup, Denmark). In brief, acetone-

fixed sections were incubated in 10% human serum in PBS for 20 minutes followed by incubation with primary antibody, diluted in PBS containing 1% bovine serum albumine (BSA), o/n at 4°C. Then, the sections were incubated with secondary antibody dilutions of biotinylated horse anti-mouse IgG, containing 10% horse serum, for 45 minutes. Staining was completed by incubation with horseradish peroxidase-conjugated avidin-biotin-complex (VectaStain ABC Elite Kit, Vector Laboratories, Burlingame, CA, USA), which was visualized with DAB (Sigma Laboratories, St. Louis, MO, USA). Sections were counterstained with haematoxylin for 30 seconds, dehydrated and mounted in entallan. Nine MS lesions were characterized as chronic active, by presence of HLA-positive foamy macrophages in the rim surrounding the gliotic centre of the lesion, and 10 lesions were chronic inactive, as determined by absence of foamy macrophages. MS lesions and sections (8 µm) of cortical grey matter and spinal cord derived from healthy donors were also stained for CD200 (clone OX104, hybridoma was kindly provided by Prof. A.N. Barclay) and CD47 (Serotec).

Laser dissection microscopy

To dissect immunohistochemically defined sub-regions of MS lesions, two series of 10 sections (20 µm each) were mounted on PALM MembraneSlides (P.A.L.M. Microlaser Technologies AG, Germany) and dried 48 h at RT, in a sealed box containing silica gel. Sections preceding and following these series were stained for HLA and PLP to visualize possible alterations in the topography of the lesion (Fig. 1A, B).

Using a laser dissection microscope (LDM; Zeiss, Oberkochen, Germany), elements that represented (parts of) the centre or rim of both chronic active and inactive lesions, as well as normal appearing white matter (NAWM) surrounding the lesions were cut and isolated from the dried, unstained sections. The same elements were cut in each of the twenty sections, with slight adjustment to the elements to match alterations in lesion topography. Afterwards, sections were stained in thionine to confirm that isolated areas were properly dissected (Fig. 1C). In control white matter, comparable elements were cut as those taken from NAWM from MS patients. The isolated tissue fragments were placed directly into TRIZOL (Invitrogen Life Technologies, Carlsbad, CA, USA) such that from each control subject one sample was obtained containing white matter, and from each MS patient three samples were obtained representing precisely isolated material from centre and rim of the lesion as well as NAWM.

Table 1. Clinical and neuropathological data of donors

Patient	Sex	Age	PM time	Duration	MS type	Lesion type	Cause of death
1	m	46	3:45	23	SP	chronic active	pneumonia
2	f	40	7:00	11	SP	chronic active	aspiration pneumonia
3	f	71	8:20	24	ND	chronic active	pneumonia
4	f	69	13:20	26	RR	chronic active	probable viral infection
5	f	48	8:10	8	SP	chronic active	euthanasia
6	f	53	10:45	27	SP	chronic active	euthanasia
7	f	66	6:20	15	SP	chronic active	pneumonia
8	f	50	7:45	17	SP	chronic active	euthanasia
9	f	43	10:45	23	SP	chronic active	subdural heamatoma, pneumonia
10	f	71	10:25	24	SP	Inactive	respiratory insufficiency
11	f	52	8:25	22	SP	Inactive	respiratory failure/pneumonia
12	f	66	6:20	43	SP	Inactive	metastases and liver failure
13	f	75	8:00	42	SP	Inactive	pneumonia
14	m	77	4:15	26	PP-SP	Inactive	CVA
15	f	71	10:15	23	SP	Inactive	post-surgery respiration problems
16	f	48	4:50	25	SP	Inactive	euthanasia
17	f	55	17:00	19	SP	Inactive	possible CVA
18	f	68	7:30	39	SP	Inactive	bronchitis/ aspiration pneumonia
19	m	70	7:45	46	ND	Inactive	cardiac arrest
20	m	75	7:15	-	-	-	heart failure
21	f	80	6:15	-	-	-	dehydration
22	f	81	22:15	-	-	-	coronary shock
23	f	54	8:00	-	-	-	acute renal failure
24	f	71	4:50	-	-	-	cardiac arrest
25	f	65	12:50	-	-	-	cardiac arrest
26	f	55	5:35	-	-	-	intracerebral bleeding
27	m	48	5:30	-	-	-	euthanasia
28	f	64	6:00	-	-	-	cachexia
29	f	41	13:30	-	-	-	massive lung bleeding

PM time = post-mortem delay until end of autopsy expressed in hours:minutes; Duration = disease duration in years; MS type = type of MS at time of death; SP = secondary progressive; RR = relapsing-remitting; PP = primary progressive; ND = not determined; CVA = cerebrovascular accident.

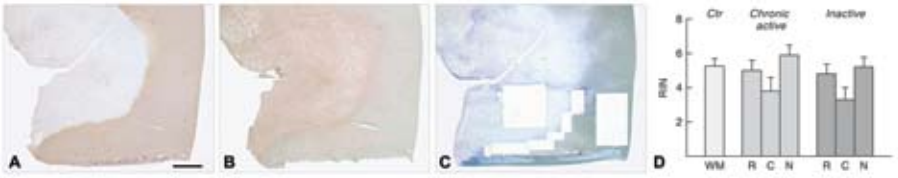


Fig. 1 Dissection of specific MS lesion areas using laser dissection microscopy. To determine immune activity and topography of the lesions, staining was performed for PLP (A) and MHC class II (B). Per patient, 2 series of 10 adjacent unstained sections (20 µm) were used to laser-dissect parts of the rim, centre and normal appearing matter of the lesion. After laser dissection microscopy, the lesion was stained with thionine (C). Quality of RNA, isolated from control white matter (WM) and from the rim (R), centre (C) and NAWM (N) of chronic active and inactive lesions, was indicated by the RNA integrity number (RIN) (D). Results are expressed as mean ± SEM, bar is 2 mm.

RNA isolation and reverse transcription

Following removal of the PALM membrane, chloroform was added to the TRIZOL lysate. An equal volume of ethanol was added to the collected aqueous phase, and this mixture was loaded onto an RNeasy Mini Kit column (Qiagen, Hilden, Germany). Further RNA isolation was performed according to the RNeasy protocol (RNeasy Mini Handbook 06/2001) and samples were stored at -80°C. RNA quantity and quality control was performed using a NanoDrop (ND-1000, NanoDrop Technologies, Rockland, DE, USA) and Bioanalyzer (2100, Agilent Technologies, Palo Alto, CA, USA), which allowed RNA quality assessment using the RNA integrity number (RIN), ranging from 1 (worst quality) to 10 (best quality).

On average 9 mm^2 brain tissue per lesion sub-area per section was isolated. The amount of total RNA isolated per sub-area from 20 sections ranged from 350 ng to 2630 ng. The pH of the cerebrospinal fluid (CSF), that can reflect post-mortem processes that might affect the quality of RNA, was significantly different ($p < 0.05$) between control subjects (pH = 6.75) and MS patients from which inactive lesions were derived (pH = 6.28). However, average RIN was 5.3 in control white matter and did not differ significantly from RIN values from rim, centre or NAWM of chronic active and inactive lesions (Fig. 1D). No correlations were found between these RIN values and qPCR results (see below for statistics).

Aliquots of 100 ng total RNA per area of nine chronic active lesions were pooled, and similarly from ten chronic inactive lesions and ten white matter controls, resulting in 0.9 µg total RNA per rim, centre and NAWM from chronic active lesions and 1.0 µg total RNA per area from chronic inactive lesions and from controls. Reverse transcription was performed in a reaction mixture of 25 µl containing 900-1000 ng total RNA, 500 µg/µl oligo(dT) and random hexamer primers (40:1) for 10 min at 80°C and 200 U/µl SuperScript II RT (Invitrogen), first strand buffer, 100 mM

dithiothreitol (DTT) and 10 mM dNTP, incubated for 1h at 42°C. All cDNA samples were diluted to a concentration of 2 ng/µl of initial total RNA and stored at -20°C, until used for further analysis.

Real-time quantitative PCR

Primer pairs for real-time quantitative PCR (qPCR) (Suppl. Table 1) were designed using the PrimerS software package for qPCR primer design (developed by R.M.H.) and specificity was tested on cDNA from brain and tonsil by assessment of the dissociation curve and PCR product as determined by size fractionation on an 8% SDS-PAGE gel. The qPCR was performed with SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) with samples containing equal cDNA concentrations of 10 ng initial total RNA per reaction. Analysis was performed according to manufacturer's protocol and the ABI Prism 7300 Sequence Detection System (Applied Biosystems). Target genes were normalized to 18S ribosomal RNA. For each primer pair, the primer efficiency (Effpr) was calculated using LinRegPCR software.[145] Fold differences were calculated by $(Effpr)^{-\Delta\Delta CT}$ method.[146]

Statistical analysis

Statistical analysis was performed using the non-parametric Mann-Whitney U test and the Spearman's correlation test. Based on the resolution of the qPCR technique, differences in gene expression ≥ 2.0 fold were considered reliable (see discussion). P-values ≤ 0.05 were considered significant.

Results

Gene expression in chronic active lesions

We measured the expression levels of 41 genes involved in macrophage regulation or in other processes expected to be important for lesion formation, on specific micro-locations within chronic active lesions. All gene expression data are presented relative to non-MS control white matter and details are listed in Supplemental Table 2.

Centre

As indicators of demyelination, axonal damage and gliosis, the hallmarks of MS, we measured the mRNA expression of myelin basic protein (MBP), neurofilament (NF) and glial fibrillary acidic protein (GFAP), respectively. MBP and NF expression was decreased in the centre of the lesion, and GFAP levels were increased (Suppl. Table

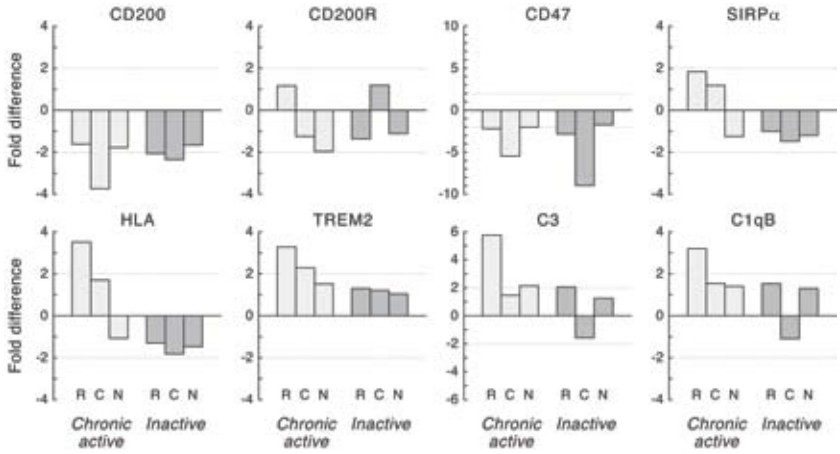


Fig. 2 Gene expression of macrophage inhibitory and activating molecules in micro-locations of MS lesions. The expression of CD200, CD200R, CD47, SIRPα, MHC class II, TREM2, C3 and C1qB was measured in pooled mRNA from the rim (R), centre (C) and NAWM (N) from chronic active and inactive MS lesions and compared to control white matter. Dotted lines indicate a two-fold difference (see Methods and Discussion sections).

2). Of macrophage activation markers, only triggering receptor expressed on myeloid cells 2 (TREM2) expression was found to be increased (Fig. 2). Further activity of the immune system was indicated by the increased expression of the chemo-kines CCL3 and CCL5 and the chemokine receptor CCR5. However, the cytokine profile was anti-inflammatory, as IL-10 expression was high and that of IL-12 and IL-23 was low compared to controls (Fig. 3). Expression of both immune suppressive molecules CD47 and CD200 was decreased, whereas expression of their receptors SIRPα and CD200R was not altered (Fig. 2). Remarkably, the expression of two molecules that are suggested to play a role in lesion development, αB-crystallin and osteopontin, were decreased. Furthermore, expression of the glucocorticoid receptor (GR) was decreased and expression of the estrogen receptor α (ERα) was highly increased.

Rim

In the rim of chronic active lesions, NF and MBP expression was decreased and that of GFAP was increased, like in the centre of the lesions. The hallmark of chronic active lesions is that the rim contains highly activated macrophages. Indeed, we found increased levels of HLA, CD11b, DAP12 and TREM2 (Fig. 2). Also, expression of the complement factors C3 and C1qB was increased (Fig. 2). The expression of CD47 was decreased, whereas expression of CD200 was not different from controls and expression of their receptors was unaltered as well (Fig. 2). The higher immune activity was further reflected by the expression of chemokines (CCL3, CCL5 and

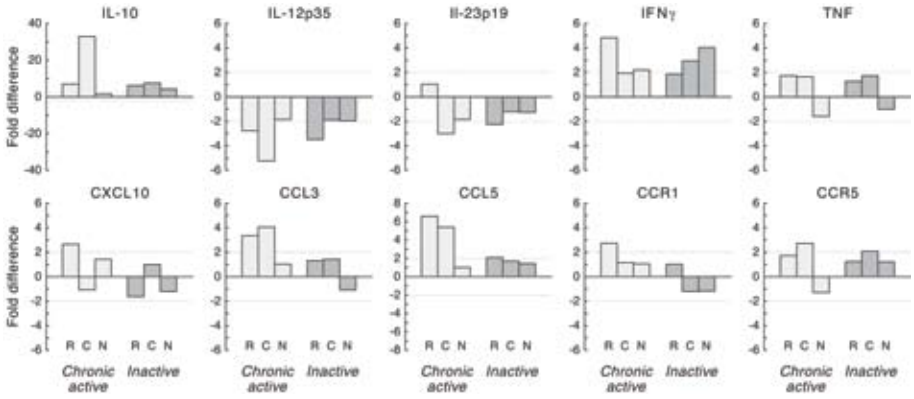


Fig. 3 Gene expression of cytokines, chemokines and chemokine receptors in micro-locations of MS lesions. Expression of IL-10, IL-12p35, IL-23p19, IFN-γ, TNF, CXCL10, CCL3, CCL5, CCR1 and CCR5 was measured in pooled mRNA from the rim (R), centre (C) and NAWM (N) from chronic active and inactive MS lesions and compared to control white matter. Dotted lines indicate a two-fold difference (see Methods and Discussion sections).

CXCL10), chemokine receptor (CCR1) and of the cytokine IFN-γ, although, similar to the centre of the lesions, IL-10 expression was increased and IL-12 expression decreased (Fig. 3).

NAWM

Only few molecules were found to be differentially expressed in the NAWM surrounding chronic active lesions. Remarkably, expression of NF and MBP were again decreased relative to controls. However, GFAP expression was not altered. Complement factor C3 expression was increased and expression of CD47 was decreased (Fig. 2). Furthermore, IFN-γ expression was increased and that of IL-17 was decreased.

Gene expression in inactive lesions

Gene expression profiles were also determined on micro-locations of inactive lesions, where macrophages have ceased their (phagocytic) activity. Therefore, the rim of an inactive lesion is a sharp boundary between the gliotic tissue and surrounding normal appearing white matter. Results of the gene expression profile analysis in inactive lesions are listed in Supplemental Table 2.

Centre

In the gliotic centre of inactive lesions, NF and MBP expression was decreased and GFAP was increased. The expression levels of CD47 and CD200 were decreased. Expression of their receptors was not different from controls, neither was the expres-

sion of molecules involved in macrophage activation (Fig. 2). Low immune activity was found as only CCR5 and IFN-γ expression was increased. Like in chronic active lesions, IL-10 expression was highly increased (Fig. 3). Also comparable to the centre of chronic active lesions was the decreased expression of GR, αB-crystallin and osteopontin and the increased expression of ERα.

Rim

In the rim of inactive lesions, NF and MBP expression was decreased and GFAP expression was increased. Also, the expression of both CD47 and CD200 was decreased and complement factor C3 expression was increased (Fig. 2). Further indication of activity of the immune system was restricted to increased CCL5 expression. Similar to the centre of chronic active and inactive lesions, IL-10 expression was high, whereas that of IL-12 and IL-23 was lower compared to controls (Fig. 3). We detected decreased expression of αB-crystallin, osteopontin and GR, and an increased expression of ERα.

NAWM

In contrast to the NAWM adjacent to chronic active lesions, NF expression was not altered. Also GFAP expression was comparable to controls, but MBP expression was decreased. No change in expression was found for CD200, CD47, CD200R and SIRPα (Fig. 2). In this area, expression of IL-10 and IFN-γ was increased (Fig 3.). Like other sub-areas of inactive lesions, expression of GR and osteopontin was decreased and that of ERα was increased.

T cell transcripts are hardly detectable in chronic active and inactive MS lesions

To determine the contribution of T cells in these lesions, we included measurement of the T cell marker CD3. In controls, rim of inactive lesions or NAWM of both types of lesions, CD3 was not detected (Ct>40). Only very low abundant transcript levels were detected in the rim of chronic active lesions (Ct = 36.23), and in the centre of both chronic active and inactive lesions (Ct = 34.86 and 38.03, respectively).

Individual gene expression closely resembles pooled mRNA data

The amount of RNA obtained from LDM-dissected lesion areas was insufficient to 1) repeatedly measure each gene, and 2) to determine the expression of every gene on each individual patient. To address these issues, the expression of a few selected genes (CD200, CD200R, CD47, SIRPα and osteopontin) was repeatedly measured in the sub-areas of 4 chronic active and 4 inactive individual lesions, and were compared with 4 controls. Expression of these genes was also determined on

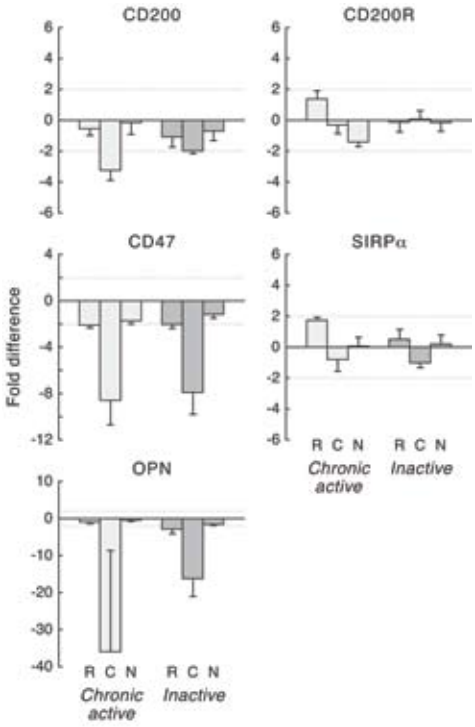
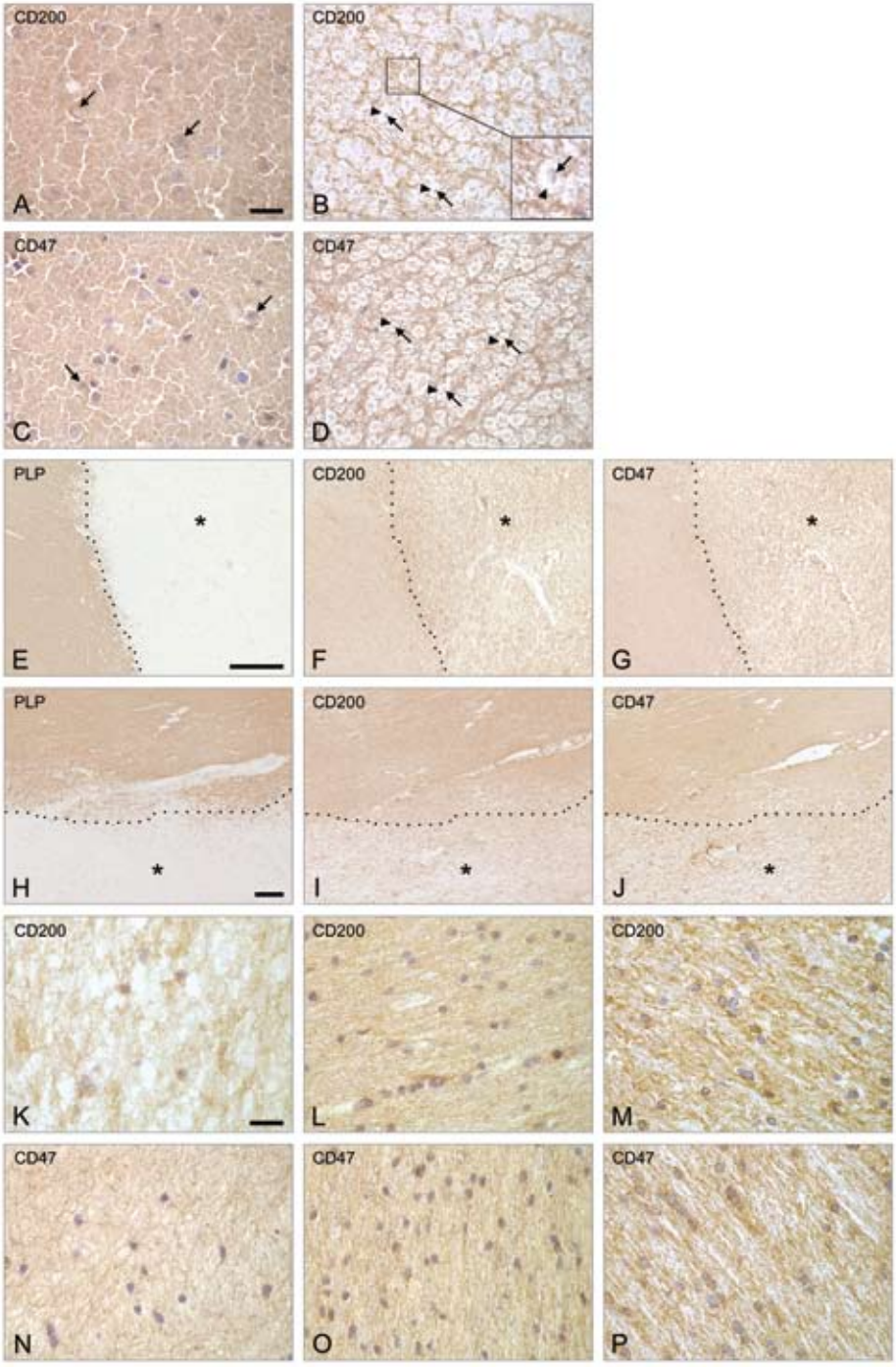


Fig. 4 Gene expression of CD200, CD200R, CD47, SIRPα and osteopontin (OPN) in MS lesions. Gene expression was determined in three experiments. In the first experiment gene expression was measured in pooled RNA per sub-area of all chronic active and all inactive lesions (see Fig. 1). In the second experiment expression was determined in 4 individual chronic active and 4 inactive lesion areas (mean of 3 measurements) and in the third experiment expression was determined in pooled cDNA of lesion sub-areas of these 4 individual chronic active and inactive lesions (mean of 2 measurements). Data are expressed as mean of the three experiments ± SEM.

pooled cDNA from each sub-area from these individuals. Low variability between the experiments was found, as shown by the combined data of gene expression in individual patients, in the pool of individual patients and in the pool of all patients (Fig. 4). Furthermore, individual values confirmed the data of these genes obtained from the pool of all patients, with no significant change in expression of CD200R and SIRPα, and a significant decrease in expression of CD200 ($p<0.05$), CD47 ($p<0.05$) in the centre of chronic active and inactive lesions (Suppl. Fig. 1). The expression of CD47 tended to be decreased in the rim of chronic active lesions and the NAWM surrounding it, but in these 4 individuals, the difference was not significant. Osteopontin expression was decreased in the centre of chronic active and inactive lesions ($p<0.01$) and also in the rim of inactive lesions ($p<0.05$). As these data are contradicting previously published data on osteopontin expression,[147,148]


CD200
A
CD200
B
CD47
C
CD47
D
PLP
E
CD200
F
CD47
G
PLP
H
CD200
I
CD47
J
CD200
K
CD200
L
CD200
M
CD47
N
CD47
O
CD47
P

we measured osteopontin expression with a second primer set that was used in the study of Tajouri *et al*. Again, in our tissue samples, osteopontin expression was decreased to a comparable extent as determined with the first primer set (data not shown).

Protein expression of CD200 and CD47 is down-regulated in MS lesions

We stained sections of 6 chronic active and 6 inactive lesions with CD200 and CD47 mAb to investigate protein expression in MS lesions. Immunoreactivity to these molecules was high in the CNS, with the most intense expression mainly found in grey matter, confirming previous findings.[120] Neuronal cell bodies were found positive in cortical grey matter from controls (Fig. 5A, C). Transverse sections of spinal cord from controls show immune-reactivity for both CD200 and CD47 on axons. No reactivity was present on compact myelin sheaths, but structures surrounding them are positive (Fig. 5B, D). On low magnification a chronic active and inactive lesion, respectively, was visualized by PLP staining (Fig 5E, H). Expression of both CD200 and CD47 was diminished within the centre of chronic active (Fig. 5F, G) and inactive MS lesions (Fig. 5I, J) when compared to the surrounding white matter. Note that RNA expression of CD200 and CD47 was compared to white matter from controls. CD200 and CD47 staining is also shown on higher magnification in the centre of a lesion (Fig. 5K, N), NAWM surrounding the lesion (Fig. 5L, O) and in white matter from control subjects (Fig. 5M, P).

Discussion

In the present study, we analyzed sub-areas of MS lesions, isolated by immunohistochemically-guided laser dissection microscopy. This technique is very precise, but the amount of RNA that can be isolated from these small tissue samples is

< Fig. 5 Protein expression of immune suppressive molecules. Sections of cortical grey matter derived from controls show CD200 (A) and CD47 (C) positive neurons (arrows). Furthermore, the surrounding tissue is also intensely stained. Transverse sections of the spinal cord from control subjects were stained for CD200 (B) and CD47 (D). Axons (arrows) are weakly positive for CD200 (enlarged in the inset), and are more intensely stained for CD47. The compact myelin sheaths surrounding the axons are unstained (arrowheads), but structures surrounding the myelin are positive. Bar is 25 µm. Low magnification of sections of a chronic active MS lesion, stained for PLP, (E) CD200 (F) and CD47 (G) shows absence of PLP intensity in the lesion center (asterisk), and presence of PLP in the NAWM at the other side of the border (dotted line) The same was done for sections of inactive lesions, stained for PLP (H), CD200 (I) and CD47 (J). In both types of lesions, the expression of CD200 and CD47 is less intense in the lesion centre than in the adjacent NAWM. Bars are 300 µm. High magnifications are shown for CD200 and CD47 respectively in the center of a lesion (K, N), the NAWM (L, O) and in white matter from controls (M, P). Bar is 25 µm. Data are representative for 6 chronic active and 6 inactive lesions.

low. Therefore it was necessary to perform the main part of this study on pooled RNA samples as this had the most important advantage that as many as 41 genes could be measured. To compensate for the lack of information on individual variability, a few genes that were considered important in this study were chosen and analyzed, using samples from individual patients and controls, on RNA that was retained after pooling the RNA samples. On the results from these experiments, statistical analysis was performed, which confirmed the findings from the data set derived from pooled RNA (Suppl. Fig. 1). Furthermore, we considered the data from real-time quantitative PCR experiments to show a reliable difference when the difference in transcript levels was at least two-fold. We chose this cut-off value because the resolution of real-time PCR is theoretically limited to a difference in cycle threshold of 1, which corresponds to a 2-fold difference, since this technique is based on doubling the target template each cycle. However, it should be kept in mind that differential expression of a molecule by a factor less than 2 could still have a biological effect.

Figure 6 gives an overview of pathological events in well defined sub-locations in MS lesions based on the present gene expression analysis. We used chronic active lesions, defined by having a hypercellular rim consisting of foamy macrophages, and inactive lesions, in which foamy macrophages are absent. From Figures 2 and 6 it is clear that the expression levels of the macrophage specific molecules HLA, CD11b, DAP12 and TREM2 were increased almost exclusively in the rim of chronic active lesions. The expression of the astrocyte marker GFAP appeared to be increased in all areas where gliosis can be found, i.e. in the rim and centre of both chronic active and inactive lesions, as shown in Fig. 6. The growth factors BDNF and NGF were decreased or not altered, indicative of a lack of repair mechanisms.

The expression levels of pro-inflammatory chemokines and chemokine receptors confirm a prominent degree of inflammation especially in chronic active lesion areas.[1] However, the unchanged expression levels of IL-1β and TNF, the decreased expression of IL-23p19, and the high level of IL-10 suggest that the chronic active lesions used in the present study probably have gone past the peak level of inflammation. In addition, based on the CD3 expression levels, there is little evidence for the presence of T cells in these stages of MS lesions, although the expression level of IFN-γ was increased. The source for this might be either NK cells or even macrophages that have previously been reported to produce IFN-γ under inflammatory conditions.[149,150]

Although the expression patterns of MBP, NF, GFAP and growth factors, summarized in Figure 6, fit the general model on pathological events in MS lesions like demyelination, axonal damage, gliosis and lack of repair mechanisms, some

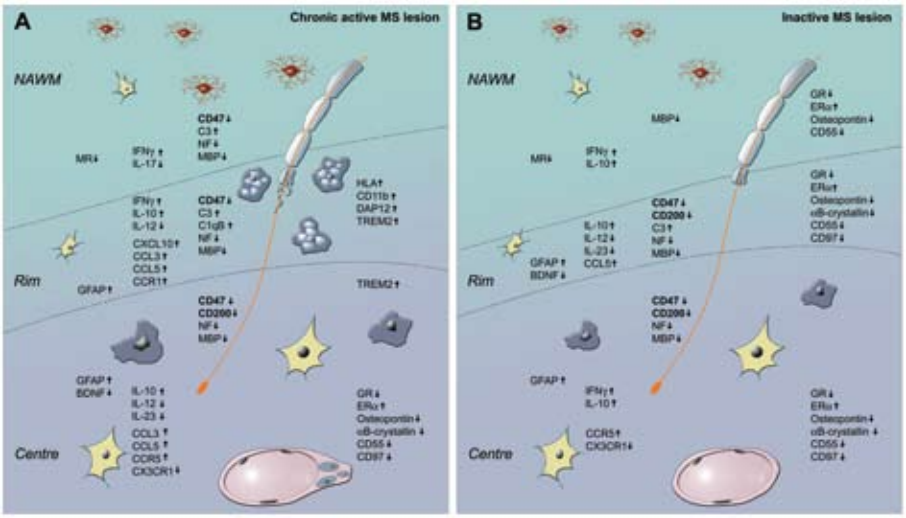


Fig. 6 Overview of gene expression profiles of genes involved in macrophage regulation, inflammation and lesion development in specific areas of chronic active (A) and inactive MS lesions (B). Expression of the immune-inhibitory molecules CD200 and/or CD47 was lower than controls in both chronic active and inactive lesions and in NAWM surrounding chronic active lesions. Most inflammatory genes were found to be expressed in chronic active lesions. Elevated expression of complement was found in the rim of both lesion types and also in NAWM around chronic active lesions. Furthermore, neuronal and oligodendrocyte-specific genes (NF and MBP, respectively) were expressed at highly reduced levels in lesions and the latter also in surrounding tissue. The astroglial gene GFAP was expressed at high levels in the lesions. Of the steroid hormones, GR expression was decreased in the centre of chronic active lesions and in all isolated regions of inactive lesions, whereas expression of ERα in these areas was increased. In the centre of both lesion types and in the inactive rim, αB-crystallin and osteopontin expression was decreased, the latter also in NAWM around inactive lesions. See text for details on the specific molecules.

remarkable differences with other studies were found.[147,148] For example, Chabas and colleagues detected in a high-throughput screening of expressed sequence tags abundant transcripts of αB-crystallin and osteopontin in two non-normalized cDNA libraries of MS tissue, which were not detected in the cDNA library of a control brain. Tajouri and colleagues detected an increase of osteopontin expression of over 50-fold in acute plaques, but a much smaller increase of up to 5-fold in chronic active plaques.[148] Acute lesions were not included in the present study, but in chronic active and inactive lesions, we detected osteopontin levels that were strongly reduced, especially in the centre of both lesion types, compared to white matter from non-MS controls (Fig. 6). These data were confirmed by a second primer pair for osteopontin that was previously used by Tajouri *et al*. The inconsistency with the former studies may be explained by the difference in techniques that were used in the study by Chabas *et al*., the smaller sample size of the other

studies, or the possibility that the control brain tissue was taken from different anatomical locations.

Steroid hormone receptors are described to have immune modulating properties in the CNS. A role for estrogens in neuroprotection has been proposed for a variety of neurological disorders.[151] Studies using MRI showed that treatment with estrogens or derivates resulted in decreased lesion numbers and volumes in MS patients[152] and decreased inflammation and demyelination in EAE.[153] Therefore it is interesting that the present study shows high expression levels of the estrogen receptor in MS lesions (Fig. 6), as these may, upon stimulation, exert beneficial effects in MS. Interestingly, GR expression was diminished in the centre of both lesion types and also in the rim and surrounding NAWM of inactive lesions. This may be a relevant finding as corticosteroids are one of the most commonly applied treatments of MS relapses.[154]

To our knowledge, this is the first study to reveal the decreased expression pattern of CD200 and CD47 in MS lesions. Importantly, expression of their receptors CD200R and SIRPα was unaltered. Biological importance of altered expression levels of molecules may be determined by several factors, for example the function of the molecule, the distribution pattern of the molecule and the possibility of the ligand to interact with its receptor. CD200 and CD47 are immune suppressive molecules that are highly abundant in the CNS. The CNS is protected by several mechanisms against harmful effects from the immune system and is thus called 'immune-privileged'. It is very likely that the presence of the normally high levels of CD200 and CD47 contribute to the immune-privileged state of the CNS. CD200 and CD47 can specifically inhibit macrophage/microglia activity. Absence of either CD47 or SIRPα in the presence of activating signals via opsonization increases phagocytic activity of macrophages.[115,144] It is thus interesting that in areas where lesions can expand, like the rim of chronic active lesions and NAWM surrounding it, expression of CD47 was diminished and expression of C3, and in the rim also C1qB, was increased suggesting a shift in balance that promotes macrophage phagocytic and inflammatory activity in and around expanding chronic active lesions and thereby likely contributes to lesion expansion and axonal injury. In the rim of inactive lesions, CD47 and CD200 expression was also reduced and expression of C3 was increased. However, the finding that in the NAWM surrounding inactive lesions, expression of these molecules was restored to levels comparable to controls (Fig. 6), suggests re-establishment of an environment that suppresses macrophages in their activity and may therefore comprise a mechanism to cause lesion progression to halt. CD47 and CD200 are expressed on neurons and their receptors on microglia. This implicates that neuron-microglia interaction is important to regulate

microglia. In the absence of CD200, microglia are spontaneously activated and EAE develops more rapidly.[125] On the other hand, up-regulated CD200 expression due to altered ubiquitination in mice with a spontaneous mutation in the *Wld*^{s} gene mediates axonal protection during EAE.[129] Blocking CD200 interactions in these mice significantly worsened the disease. Not only neurons, but also glial cells may express CD47 and CD200. These cells may therefore also serve to protect neurons as they may also contribute to maintain immune suppression via these molecules. In the present study we found that in all areas where CD200 or CD47 expression was decreased, also expression of NF and MBP was decreased (Fig. 6). Loosing 2- to 9-fold of the expression of CD200 and CD47 is thus a strong indication that immune inhibition and thereby also the protection of neurons is hampered.

As mentioned before, it is highly unusual for the CNS to get inflamed due to its immune-privileged status. MS is one of the very few diseases in which CNS inflammation in the parenchyma does occur. CD200 and CD47 bind to receptors on myeloid cells, so a decreased expression of CD200 and CD47 mainly affects macrophages and microglia. As macrophages are thought to be crucial effector cells in MS, it is therefore likely that decreased myeloid cell inhibition has specific implications for this disease. Although it is unknown whether CD200 and CD47 expression is altered in other human CNS disorders, studies in *Wld*^{s} mice, that have increased levels of CD200, showed that neurons are protected against several forms of axonal injury other than induced by EAE, such as Wallerian degeneration after peripheral and CNS nerve transections, and after apoptosis of the neuronal cell bodies.[155-157]

In conclusion, in this study the expression levels of multiple genes were characterized in specific MS lesion areas. The expression of the immune inhibitory molecules CD200 and CD47 is normally high in the immune-privileged CNS, but expression is decreased in MS lesions. This suggests that the balance in macrophage regulation has shifted towards activation by a hampered inhibitory input via CD200 and CD47 in areas where myelin and axonal damage are found. Combined with presence of immune activating molecules, this microenvironment may promote ongoing inflammation around chronic active lesions with irreversible axonal damage as a consequence and a possible enhancement of phagocytic activity that can lead to expansion of the lesion. In terms of therapeutic interventions we show that expression of the receptors SIRPα and CD200R was not changed, making them a potential target for artificial ligand ligation to maintain or restore the balance in macrophage inhibition and to halt inflammation and possibly phagocytosis early in lesion development, thereby preventing the extension of axonal injury.

Acknowledgments

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Supplemental data

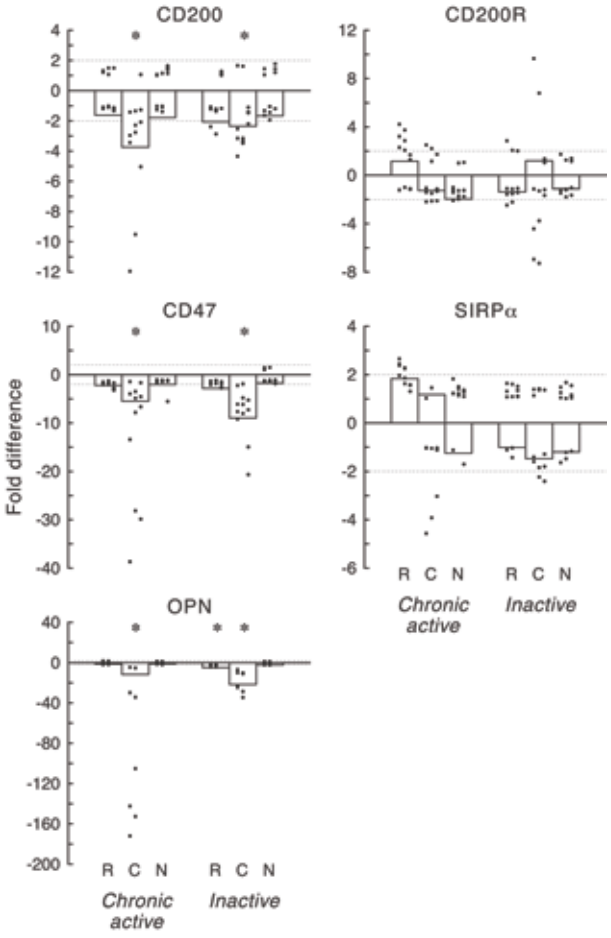

Fig. 1 Gene expression in 4 chronic active and 4 inactive MS lesions. Individual gene expression data are shown for CD200, CD200R, CD47 and SIRPα (3 experiments) and for osteopontin (OPN) (2 experiments). The bar represents the value obtained from the pooled sub-areas of chronic active and inactive lesions. Significant changes are indicated with *.

Table 1. Primer sequences

Protein	Database no	Primer sequence forward	Primer sequence reverse
HLA-DR	NM_019111	CCCAGGGAAGACCACCTTT	CACCCTGCAGTCGTAAACGT
CD11b	NM_000632	TGCTTCCTGTTTGGATCCAACCTA	AGAAGGCAATGTCACTATCCTCTTGA
DAP12	NM_003332	GGGTCAGAGGTCGGATGTCTA	AGGAATGGCTGGATCCAGGTAT
TREM2	NM_018965	CCACCCACTTCCATCCTTCT	GTCCCTGGCTTCTGTCCAT
CD55	NM_00574	GGGCAGTCAATGGTCAGATATTGA	GGCTGTTTGAGGGATGCAGAAT
CD97	NM_078481	GGTGCTGACCTATGTGTTTACCA	AGGCCCACTTCCGGTATTCTT
CD200	NM_005944	CCAGGAAGCCCTCATTGTGA	TCTCGCTGAAGGTGACCATGT
CD47	NM_001777	ATGGAGCTCTAAACAAGTCCACTGT	TGTGAGACAGCATCACTCTTATCCAT
CD200R	NM_138806	GAGCAATGGCACAGTGACTGTT	GTGGCAGGTCACGGTAGACA
SIRPα	NM_080792	GTCTGGAGCAGGCACTGA	GGACTCGCAGGTGAAGCT
TGFβ2	NM_003238	GCTGGAGCATGCCCGTATTTAT	CGCAGCAAGGAGAAGCAGAT
IL-1β	NM_000576	CCGACCACCACTACAGCAA	GGCAGGGAACCAGCATCT
IL-17	NM_002190	CAACCGATCCACCTCACCTT	GCACTTTGCCTCCCAGATCA
TNF	NM_000594	GGCGTGGAGCTGAGAGATA	CAGCCTTGGCCCTTGAAGA
IFN-γ	NM_000619	GCAAGATCCCATGGGTTGTGT	CTGGCTCAGATTGCAGGCATA
IL-10	NM_000572	TGCCTTCAGCAGAGTGAAGACTT	TCCTCCAGCAAGGACTCCTTTA
IL-12 p35	NM_000882	GGAGGCCTGTTTACCATTGGA	GGCCAGGCAACTCCCATTA
IL-23 p19	NM_016584	GGATCCACCAGGGTCTGATT	CCCAGTAGGGAGGCATGA
IP-10	NM_001565	TACGCTGTACCTGCATCAGCATT	GCAATGATCTCAACACGTGGACAAA
CCL3	NM_002983	GCAGCAGACAGTGGTCAGT	GTGCAGAGGAGGACAGCAA
CCL5	NM_002985	TCTGTGACCAGGAAGGAAGTCA	GAGACGGCGGAAGCTTAAGA
CCR1	NM_001295	ACGGAGGTGATCGCCTACA	AACAACTGCCGCAGGTACTT
CCR5	NM_000579	TCTTCTGGGCTCCCTACAACATT	CTGCATAGCTTGGTCCAACCT
CX3CR1	NM_001337	TTGGCCTGGTGGGAAATTTGT	AGGAGGTAAATGTCGGTGACACT
Fractalkine	NM_002996	CAGGCCACAAGACCCTTGT	GCCCAGCCTCAGGAAAGAAT
SEMA 3A	NM_006080	CCTTACTGTGCTTGGGATGGT	AGTGAGTCAGTGGGTCTCCAT
MR	NM_002438	TGCAGAAGCAAACCAAACCT	CAGGCCTTAAGCCAACGAAACT
GM-CSF	NM_000758	CCCGGAAACTTCCTGTGCAA	CTGGCTCCCAGCAGTCAA
NGF	NM_002506	TGTACCACGACTCACACCTTTGT	CACAGGCCGTATCTATCCGGATAA
BDNF	NM_170735	GGGACGGTCACAGTCCTT	CCCATGGGATTGCACTTGGT
c-KIT	NM_000222	TGCACTGATCCGGGCTTT	TGTTGGTGGCTTCTGCCTTT
NF	NM_021076	CCCAGCTGCGAGAATACCA	CCAAAGCCAATCCGACACTCT
MBP	NM_001025081	GGGTCTTCCTGGAGATTTGGT	GCTGTGGTTTGGAAACGAGGTT
αB-crystallin	NM_001885	GTCCTCACTGTGAATGGACCAA	GGTGACAGCAGGCTTCTCTT
Osteopontin	NM_001040058	CGAGGACATCACCTCACACA	CCACGGCTGTCCCAATCA
GFAP	NM_002055	CCGCCACTTGCAGGAGTA	GGGAATGGTGATCCGGTTCT
BZRP	AY998017	TACCGTGGCCTGGTACCA	TCCCGCCATACGCAGTAGTT
GR	NM_001018077	GCCAAGGATCTGGAGATGACAA	GAGGAGAGCTTACATCTGGTCTCA
ERα	NM_000125	ACGGTTCAGATAATCCCTGCTG	CCATTGGTGTTGGATGCATG
C3	NM_000064	CGGACGGTCATGGTCAACAT	ATGTCCCAAGACAAGGGCAAGA
C1qB	NM_000491	CCGCTTCGACCACGTGAT	AGTAGAGACCGGGCACCTT

Table 2. Fold change in gene expression in MS lesions relative to control white matter

	Chronic active lesion			Inactive lesion		
	Rim	**Centre**	**NAWM**	**Rim**	**Centre**	**NAWM**
HLA	3.51	1.70	-1.07	-1.30	-1.82	-1.47
CD11b	2.25	1.55	-1.11	1.00	-1.11	-1.40
DAP12	3.34	1.26	-1.04	-1.08	-1.81	-1.20
TREM2	3.28	2.29	1.52	1.30	1.21	1.04
CD55	-1.94	-5.82	-1.70	-3.14	-11.34	-2.14
CD97	-1.03	-2.58	-1.55	-2.81	-8.26	-1.95
CD200	-1.62	-3.74	-1.78	-2.06	-2.35	-1.66
CD47	-2.21	-5.48	-2.02	-2.83	-8.97	-1.73
CD200R	1.16	-1.25	-1.96	-1.37	1.19	-1.11
SIRPα	1.84	1.18	-1.25	-1.01	-1.48	-1.20
TGFβ2	1.65	-1.11	-1.04	1.09	-1.19	-1.14
IL-1β	1.04	-1.33	-1.35	-1.14	1.18	1.07
IL-17	-1.09	-1.18	-2.03	-1.51	1.35	-1.43
TNF	1.73	1.66	-1.58	1.28	1.73	-1.01
IFN-γ	4.82	1.93	2.19	1.85	2.94	4.04
IL-10	7.03	32.93	1.71	6.18	7.59	4.31
IL-12	-2.77	-5.23	-1.84	-3.48	-1.87	-1.96
IL-23	1.05	-3.00	-1.84	-2.24	-1.20	-1.24
IP-10	2.66	-1.07	1.43	-1.62	1.00	-1.19
CCL3	3.36	4.06	1.03	1.32	1.43	-1.08
CCL5	6.58	5.39	1.00	2.10	1.70	1.43
CCR1	2.74	1.16	1.05	1.02	-1.19	-1.16
CCR5	1.74	2.73	-1.28	1.25	2.08	1.21
CX3CR1	-1.43	-2.01	-1.40	-1.77	-2.76	-1.68
Fractalkine	1.66	1.07	-1.14	1.34	1.38	1.20
SEMA 3A	1.25	1.13	1.24	1.29	1.43	1.30
MR	-1.05	-1.25	-2.77	-1.81	1.38	-3.63
GM-CSF	1.49	-1.49	-1.19	-1.46	1.34	1.02
NGF	1.08	-1.63	-1.19	-1.44	1.23	-1.21
BDNF	1.00	-3.63	-1.52	-2.65	-1.58	-1.63
c-KIT	-2.42	-2.25	-2.85	-2.72	-1.82	-1.67
NF	-2.33	-2.54	-4.69	-7.27	-5.05	-1.96
MBP	-3.94	-59.53	-2.04	-3.56	-142.33	-2.15
αBcrystallin	-1.89	-5.13	-1.98	-2.03	-4.52	-1.50
Osteopontin	-1.22	-11.64	-1.11	-4.88	-21.57	-2.10
GFAP	3.08	3.60	1.48	2.74	3.16	1.86

	Chronic active lesion			Inactive lesion		
	Rim	**Centre**	**NAWM**	**Rim**	**Centre**	**NAWM**
BZRP	1.24	1.24	-1.24	-1.09	-1.40	-1.12
GR	-1.79	-4.23	-1.74	-4.23	-7.11	-2.08
ERα	1.89	18.57	1.40	9.02	7.40	6.54
C3	5.77	1.47	2.14	2.06	-1.57	1.25
C1qB	3.20	1.54	1.40	1.53	-1.09	1.30

3

CD200 and CD200R localization in the brain

Distribution of the immune inhibitory molecules CD200 and CD200R in the normal central nervous system and multiple sclerosis lesions suggests neuron-glia and glia-glia interactions

Nathalie Koning, Dick F. Swaab, Robert M. Hoek, Inge Huitinga

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Abstract

CD200 is a membrane glycoprotein that suppresses immune activity via its receptor, CD200R. CD200-CD200R interactions have recently been considered to contribute to the 'immune-privileged' status of the central nervous system (CNS). The mechanisms by which these interactions take place are not well understood as detailed reports on the distribution of CD200 and CD200R are lacking. We used immunohistochemistry to characterize the distinct anatomical and cellular distribution of these molecules in multiple sclerosis (MS) lesions and controls. CD200 was robustly expressed in grey matter CNS areas including the cerebral cortex, hippocampus, striatum, cerebellum and spinal cord, where neurons appeared immunopositive. CD200 expression was also detected in oligodendrocytes, but not in astrocytes or microglia. In CNS samples from MS patients, CD200 expression was additionally observed on reactive astrocytes in chronic active lesions. This was an interesting finding as we previously showed that overall CD200 expression in MS lesions was decreased. In contrast to CD200, the localization pattern of CD200R was very distinct, showing high expression on perivascular macrophages in both grey and white matter. Using flow cytometry we show that human primary microglia express low levels of CD200R. These data suggest that CD200-mediated immune suppression may occur not only via neuron-microglia interaction, but also via glia-glia interactions, especially in inflammatory conditions in which an immune-suppressed environment needs to be restored, possibly by enhancing CD200 expression.

Introduction

A sensitive organ like the central nervous system (CNS) necessitates a shelter from peripheral immune responses to protect its cells that have low regenerative capacity. Several strategies have thus evolved to create an 'immune-privileged' environment, from which the mechanisms are only partly understood. The blood-brain barrier, which restricts the entrance of leukocytes into the CNS, is one of the best studied systems. Furthermore, communication deficiency between the CNS and the peripheral immune system is known to involve the afferent arm of the immune system and is established by the lack of competent antigen presenting cells and a conventional lymphatic system.[158] However, neurons, that hardly regenerate, not only need to be protected from the peripheral immune system, as microglia, the resident macrophages of the CNS, are present throughout the CNS and need to be continually constrained as well. Therefore, a highly important, but less well studied mechanism to protect the CNS against both peripheral and central immune responses is the presence of molecules that directly inhibit immune cells. An example of such a molecule is CD200, which is highly expressed on neurons and can down-regulate immune activity through binding with its receptor, CD200R.[121,125,159] That CD200-CD200R interaction in the brain contributes to the immune-privileged status of the CNS is supported by the observation that in other immune-suppressed organs like the placenta, CD200 expression is also remarkably high.[120]

CD200 and its receptor are homologous membrane glycoproteins belonging to the immunoglobulin superfamily. CD200 is expressed on numerous cell types in a wide variety of tissues[120] whereas in man CD200R is mainly expressed on myeloid cells like monocytes and macrophages and on a subset of T and B cells.[122,124] In rats, CD200R has been demonstrated on microglia as well.[121,160] Unlike its ligand, CD200R has signaling motifs in its intracellular domain that upon triggering deliver an inhibitory signal to the cell.[123,124] Animal studies show that CD200 is highly expressed in the CNS. When absent, mice have spontaneously activated microglia and show an enhanced susceptibility to several inflammatory disease models such as experimental autoimmune uveoretinitis (EAU) and experimental autoimmune encephalomyelitis (EAE), the animal model for multiple scherosis (MS).[125,126] Conversely, mice with inherently increased levels of CD200 due to a mutation in the *Wld*^{s} gene, have less activated and infiltrated macrophages during EAE, milder symptoms of the disease and show increased neuroprotection.[129]

Animal models thus demonstrate that loss of immune suppression via CD200 in the CNS has severe consequences for neuroinflammation and neurodegeneration. In human, activated microglia are associated with neurodegeneration in ageing, as

well as in diseases such as stroke, MS, Alzheimer's disease and Parkinson's disease. In MS, not only activated microglia but also infiltrating leukocytes cause severe inflammation leading to multiple demyelinated lesions. In a recent study, we showed that the expression of CD200, but not CD200R, was diminished in and around MS lesions and may therefore contribute to macrophage/microglia activation and inflammation in the CNS of MS patients.[111]

CD200 has been shown on neurons in the rat CNS and in the human retina,[159,160] but detailed information on CD200-CD200R expression in the human CNS is missing. With the identification of the cellular compartments that express CD200 and its receptor, we gain insight into the mechanisms by which these molecules suppress deleterious inflammatory responses in the CNS. This knowledge can help delineate the targets for therapeutic approaches to restore immune suppression when needed, for instance during MS. In this study we show in detail the broad anatomical and cellular localization of CD200 and CD200R in the human CNS, demonstrating that CD200R is expressed at low levels on human microglia and that CD200 expression is not only restricted to neurons but can also be expressed by glial cells, in particular under pathological conditions.

Methods

Tissue and donors

Snap-frozen tissue specimens of parietal cortex, hippocampus, hypothalamus, cerebellum and spinal cord of three control subjects and white matter lesions of eight MS patients obtained at autopsy were provided by the Netherlands Brain Bank, Netherlands Institute for Neuroscience, Amsterdam. Permission was obtained for brain autopsy and the use of the tissue and clinical information for research purposes. All MS patients were clinically diagnosed by a neurologist (Prof. C.H. Polman, VUMC, Amsterdam), and the diagnosis was confirmed post-mortem by a neuropathologist. Donor characteristics are shown in Table 1.

Immunohistochemistry

Cryostat sections (8 µm) were fixed in acetone for 10 minutes. Then, sections were incubated with monoclonal antibodies to CD200 (Clone OX104 for human and OX90 for mouse tissue, hybridomas were kindly provided by Prof. A.N. Barclay, University of Oxford, UK and has been described previously[120]), CD200R (Clone OX108[130], Serotec, Oxford, UK) or mannose receptor (Clone 15-2, Serotec), diluted in PBS containing 1% bovine serum albumine (BSA), o/n at 4°C. After washing

Table 1. Clinical and neuropathological data of donors

Patient	Sex	Age	PM time	Duration	MS type	Lesion type	Cause of death
1. Control	f	64	6:00	-	-	-	cachexia
2. Control	m	80	7:15	-	-	-	cachexia and dehydration
3. Control	f	82	11:30	-	-	-	congestive heart failure
4. MS	f	40	7:00	11	SP	chronic active	pneumonia
5. MS	f	50	7:45	17	SP	chronic active	euthanasia
6. MS	f	66	6:20	15	SP	chronic active	pneumonia
7. MS	f	69	13:20	26	RR	chronic active	probable viral infection
8. MS	f	68	7:30	39	SP	inactive	bronchitis/aspiration pneumonia
9. MS	f	71	10:15	23	SP	inactive	postsurgery respiratory problems
10. MS	f	75	8:00	42	SP	inactive	pneumonia
11. MS	m	77	4:15	26	PP-SP	inactive	cerebrovascular accident

PM time = post-mortem delay until end of autopsy expressed in hours:minutes; Duration = disease duration in years; MS type = type of MS at time of death; SP = secondary progressive; RR = relapsing remitting; PP = primary progressive.

in PBS, the sections were incubated with secondary antibody dilutions of biotinylated horse anti-mouse IgG (Vector Laboratories, Burlingame, CA), containing 10% horse serum, for 45 minutes, followed by 45 minute incubation in horseradish peroxidase-conjugated avidin-biotin-complex (ABC, VectaStain ABC Elite Kit, Vector Laboratories). To amplify the staining, CD200R-treated sections were incubated with biotinylated tyramine[161] in addition of 0.01% H_2O_2 for 10 minutes, after which the sections were again incubated in ABC for 45 minutes. Staining in all sections was visualized with DAB (Sigma Laboratories, St. Louis, MO) applied for 10 minutes. Sections were counterstained with haematoxylin for 30 seconds, dehydrated and mounted in entallan (Merck, Darmstadt, Germany). For fluorescent CD200 double stainings, sections were first incubated with a polyclonal antibody to glial fibrillary acidic protein (GFAP) (DakoCytomation, Glostrup, Denmark) and monoclonal antibodies to CNPase (Clone 11-5B, Sigma, St. Louis, MO), neurofilament (NF, clone SMI-311R, Covance, Emryville, CA) or FVIII (kindly provided by Dr. L. Bö, VU medical center, Amsterdam, The Netherlands) for 1 h at RT, followed by incubation with goat-anti-rabbit or goat-anti mouse Alexa 594-labeled antibody (Invitrogen-Molecular Probes, Eugene, OR) in the presence of 10% normal goat serum, for 1 h at RT. Next, sections were incubated with biotinylated anti-CD200 o/n at 4°C, followed by incubation with Alexa 488-labeled streptavidin-avidine (Invitrogen). Finally, sections were mounted in mounting medium (0.1 M Tris-HCl, 25% glycerol, 10% mowiol (EMD chemicals, Gibbstown, NJ). As negative controls, primary antibodies

were either omitted or substituted with an isotype control antibody (IgG1 or biotinylated IgG1, Serotec). All negative controls showed no immunoreactivity.

MS lesion inflammatory activity was characterized as described previously,[34,35,111,135] in cryostat sections (8 µm) stained for proteolipid protein (Serotec, Oxford, UK) and human leukocyte antigen (HLA)-DP, DQ, DR (Clone CR3/43, Dako). We used four chronic active lesions, determined by the presence of HLA-positive foamy macrophages in the rim surrounding the gliotic centre of the lesion, and four inactive lesions, as determined by absence of foamy macrophages.

Human microglia isolation

Corpus callosum white matter, provided by the Netherlands Brain Bank, was obtained at autopsy and stored in Hibernate A medium (Brain Bits LLC, Springfield, IL) at 4°C. Within 5 – 18 hours after autopsy, microglia were isolated using a Percoll (GE Healthcare, Diegem, Belgium) gradient, as described previously,[162] with some adaptations. In brief, tissue (per gram) was mechanically dissociated using a metal sieve in a total volume of 50 ml GKN/0.2% BSA (8 g/l NaCl, 0.4 g/l KCl, 1.77 g/l $Na_2HPO_4.2H_2O$, 0.69 g/l $NaH_2PO_4.H_2O$, 2 g/l D-(+)-glucose, 2 g/l BSA, pH 7.4). After centrifugation (400g, 7 min), the pellet was reconstituted in 5 ml dissociation buffer (4 g/l $MgCl_2$, 2.55 g/l $CaCl_2$, 3.73 g/l KCl, 8.95 g/l NaCl, pH 6-7) and enzymatically digested in 200 µg/ml DNase I (Roche Diagnostics, Mannheim, Germany) and 150 U Collagenase Type I (Worthington, Lakewood, NJ, USA) for 1 h at 37°C. During this incubation, the tissue was mechanically disrupted by passing the mixture through a pipet tip with decreasing bore size every 5-10 minutes. After washing in GKN/0.2% BSA, the cells were resuspended in 20 ml Percoll (ρ = 1.03), underlain by 10 ml Percoll (ρ = 1.095) and overlain by 5 ml GKN/0.2%BSA and centrifuged at 1200g for 30 min with slow acceleration and no break. The myelin layer was removed from the GKN-Percoll 1.03 interface and discarded, after which the cells from the 1.03-1.095 interface were collected. The average number of recovered cells was $3x10^6$ cells/gram tissue.

Flow cytometry

The freshly isolated CNS cells were incubated with fluorescein isothiocyanate-labeled antibody against human CD45 (Clone HI30), phycoerythyrin-labeled CD11b (Clone ICRF44, Dako) and Alexa 647-labeled CD200R (Clone OX108, Serotec) or with the appropriate isotype controls in the presence of 10% human pool serum for 1 h at 4°C. Antibody dilutions and washing steps were performed in FACS buffer (PBS, 0.2% BSA, 10 mM sodium azide). After washing, the cells were analyzed using a FACSCalibur flow cytometer (BD PharMingen, San Jose, CA) and the data were

analyzed using FlowJo software version 8.7.1 (Treestar, Inc. Ashland, OR). Cells were sorted on a FACS Aria cell sorter (BD PharMingen).

Results

Anatomical and cellular CD200 expression in the human CNS

CD200 was broadly expressed throughout the human CNS, of which Figure 1 shows examples in parietal cortex, hippocampus, striatum, cerebellum and spinal cord. The most intense expression of CD200 was present in grey matter from e.g. cortex and hippocampus, where neurons appeared CD200 positive (Fig. 1A, B, insets). Although CD200 immunoreactivity seemed present on the neuronal cell membrane, this was difficult to determine as staining was diffusely present between neuronal cell bodies and, in hippocampal neurons for example, staining seemed to be present in the cytoplasm as well (Fig. 1B). CD200 staining in the striatum showed expression in white matter, but less abundant and more distinct compared to grey matter (Fig. 1C, D). In the cerebellum, CD200 was predominantly expressed in the granular and molecular layers (Fig. 1E, F). The membranes of Purkinje neurons appeared positive and the cytoplasm was unstained (Fig. 1F inset). In agreement with our earlier report, CD200 was also present on axons (Fig. 1G, H).[111] Transversal sections of the spinal cord showed that the myelin immediately surrounding the axons was unstained, although CD200 was expressed on elements surrounding the myelin sheaths (Fig. 1G inset). This peculiar expression pattern suggests that CD200 expression was not limited to neurons and their axons. We thus analyzed CD200 expression by double staining techniques. From these it was confirmed that axons expressed CD200, as seen by colocalization with neurofilament (NF) in a longitudinal section of the spinal cord (Fig. 2A). Indeed, the fluorescent images demonstrate that CD200 expression was broad and not limited to axons. Double labeling a transversal section of the spinal cord with CNPase showed that the elements surrounding the myelin sheaths as seen in Fig. 1G were oligodendrocytes expressing CD200 (Fig. 2B). In addition, CNPase and CD200 labeling in white matter clearly showed colocalization on the oligodendrocyte cell body (Fig. 2C). However, no colocalization of CD200 with GFAP was found, showing that in the non-diseased brain, astrocytes did not express CD200 (Fig. 2D). Also microglia did not express CD200, as no colocalization with CD11b was found (data not shown). Despite the fact that CD200 expression has been described on vascular endothelial cells in the rat cerebellum[159] and in the human liver[120], we could not find CD200 colocalization with the endothelial marker Factor VIII, indicating that human brain vascular en-

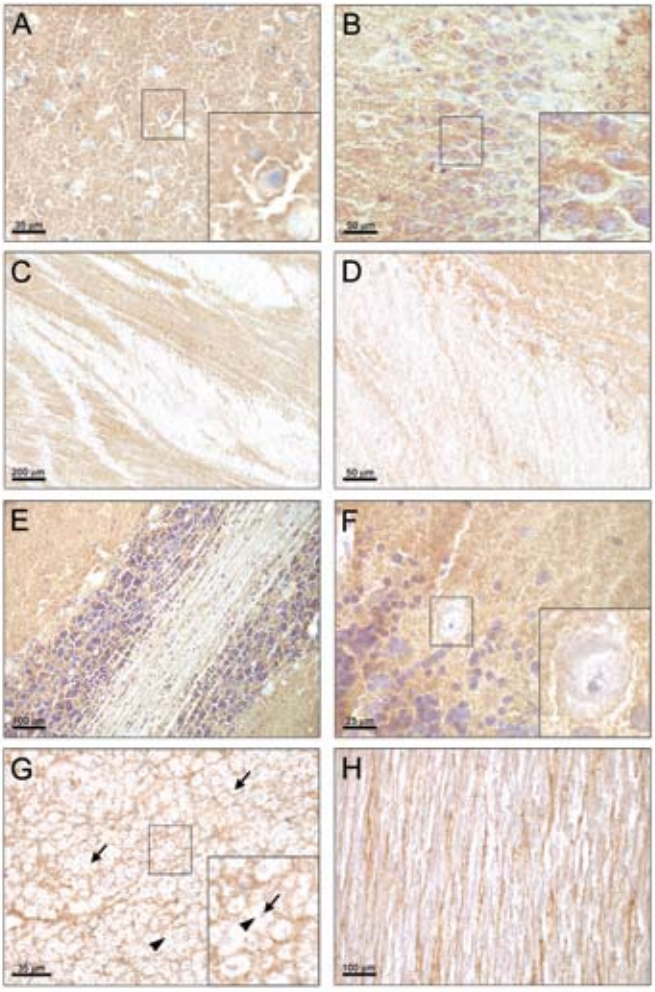

Fig. 1 CD200 expression in frozen sections (8 µm) of the human central nervous system derived from control donors. CD200 immunoreactivity is shown in parietal cortex (A), hippocampus (B), striatum (C, D), cerebellum (E, F) and in transversal and longitudinal sections of the spinal cord (G and H; respectively). CD200 is expressed on neurons (A, B, F; insets) and axons (G; arrows), but myelin is unstained (G; arrowheads). In grey matter, CD200 expression surrounding neurons is diffuse. In white matter CD200 is distinct and possibly present on non-neuronal elements.

dothelium did not express CD200 (data not shown).

CD200 expression in MS lesions

Because we previously demonstrated that CD200 expression in MS lesions was decreased, we analyzed cellular CD200 expression in MS. In and around the border of MS lesions, oligodendrocytes were CD200 positive (Fig. 3A), while staining of both

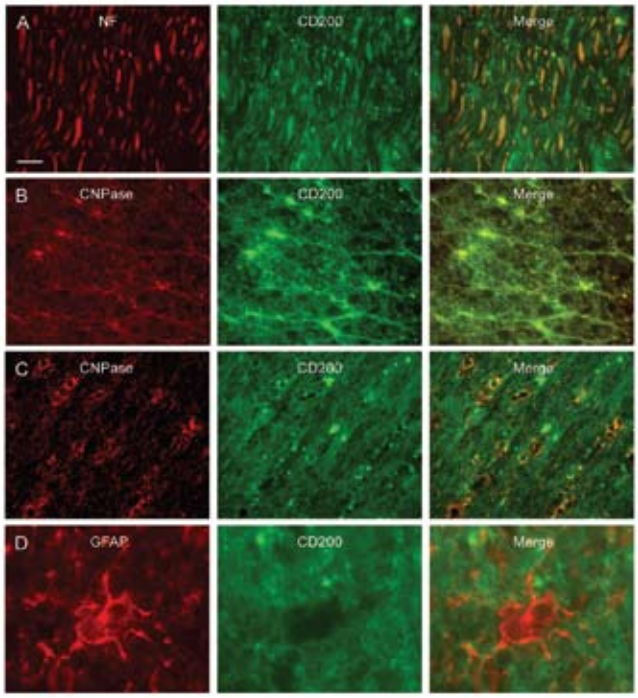

Fig. 2 Double fluorescent staining of CD200 with CNPase and GFAP in frozen human spinal cord and white matter sections (8 µm) derived from control donors. CD200 is expressed on neurofilament (NF)-positive axons shown a longitudinal section of the spinal cord (A). CD200 is expressed on CNPase-positive oligodendrocytes as shown in a transversal section of the spinal cord (B) and in white matter (C). CD200 expression is not present in GFAP positive astrocytes (D). Bar is 17.5 µm (A,B), 10 µm (C) and 5 µm (D).

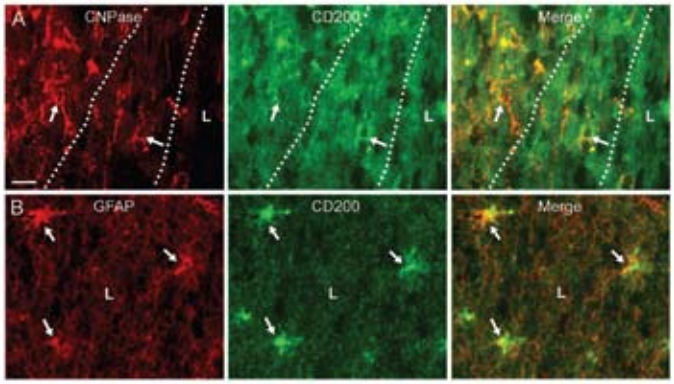

Fig. 3 Double fluorescent staining of CD200 with CNPase and GFAP in frozen sections (8 µm) of chronic active multiple sclerosis (MS) lesions. CD200 is expressed on CNPase-positive oligodendrocytes in the rim of a chronic active MS lesion (A, arrows). The area between the dotted lines is the border between the lesion (L) and the surrounding tissue. CD200 expression is present in GFAP-positive reactive astrocytes as shown in the center of a chronic active MS lesion (B, arrows). Bar is 20 µm (A) and 30 µm (B).

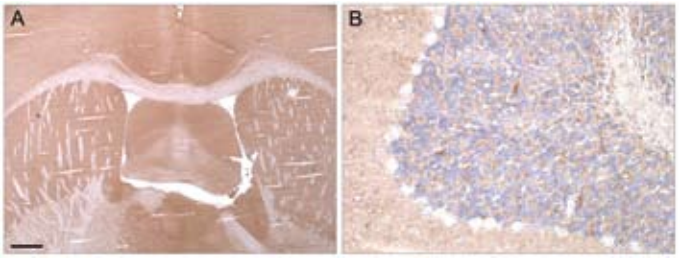

Fig. 4 CD200 expression in frozen sections (8 µm) of the mouse central nervous system. CD200 immunoreactivity is present in both grey and white matter areas, but staining intensity is low in white matter areas as corpus callosum and in the striatum compared to grey matter areas where CD200 staining is diffuse (A). CD200 staining is predominantly present in the granular and molecular layer of the cerebellum (B). Bar is 500 µm (A) and 65 µm (B).

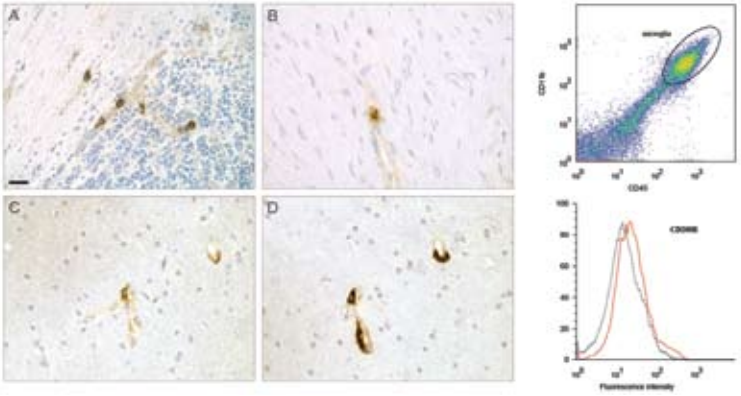

Fig. 5 CD200R staining in frozen sections (8 µm) of the human central nervous system derived from control donors. CD200R immunoreactivity is visible on perivascular cells as shown in the cerebellum (A) and in the spinal cord (B). Adjacent (8 µm) sections of white matter show CD200R expression in perivascular cells (C) and mannose receptor expression in the same cells (D). Bar is 10 µm (A-D). Human $CD45^{dim}CD11b^{+}$ microglia were isolated from corpus callosum and stained for CD200R (solid line) or an isotype-matched negative control (dotted line) (E). CD200R is present on microglia at low levels. Data are representative of 3 independent experiments.

CNPase and CD200 was diminished in the center of the lesion (data not shown, see also Koning *et al.*[111]). Unexpectedly, a subpopulation of GFAP-positive astrocytes in chronic active MS lesions also expressed CD200 (Fig. 3B). These lesion-associated astrocytes were all hypertrophic as determined by their characteristic morphology. Astrocytes situated further from the lesion did not express CD200.

CD200 expression in the normal mouse brain

Although CD200 expression has been described in the rodent brain, this has been poorly documented. In Figure 4 we show that the CD200 expression pattern in

the mouse brain was similar to that found in the human CNS. It was abundantly present throughout the brain and staining was highly intense in the grey matter. Staining was absent in CD200$^{-/-}$ mice (data not shown).[125,136,159]

Anatomical and cellular CD200R expression in the human CNS

Although CD200R immunoreactivity was present throughout the parietal cortex, hippocampus, striatum, cerebellum and spinal cord, its expression pattern was completely different from its ligand. CD200R in both grey and white matter was limited to cells associated with blood vessels (Fig. 5A, B) and staining of adjacent sections with mannose receptor (MR) demonstrated that these cells were perivascular macrophages (Fig. 5C, D).[163]

The immunohistochemical techniques we used did not show CD200R immunoreactivity on human microglia. Although studies in rodents have shown that CD200R was expressed on microglia, these were reported at low levels using flow cytometry and not supported by immunohistochemistry either.[121,136] Therefore, we analyzed CD200R expression on primary microglia, freshly isolated from human autopsy material using the highly sensitive technique of flow cytometry. We show that CD45^{dim}CD11b^{+} human microglia reproducibly expressed low amounts of CD200R (Fig. 5E), with mean fold difference of 1.6 ± 0.2 compared to isotype control. Expression of CD200R was markedly higher on choroid plexus-derived macrophages (5.3 ± 1.0) or peripheral blood monocytes (2.4 ± 0.5). In addition, gene expression analysis on these isolated microglia upon FACS sorting confirmed the presence of CD200R transcript in these cells (data not shown).[124]

In both chronic active and inactive MS lesions, CD200R expression by immunohistochemistry was clearly visible on blood vessel-associated cells that were present in all areas of the lesions and in the normal appearing white matter surrounding the lesions. In chronic active MS lesions, some perivascular cuffs contained CD200R positive cells, but others did not (Fig. 6A, B). Occasionally, CD200R expression was present on foamy macrophages, but only when located perivascularly (Fig. 6C).

Fig. 6 CD200R staining in frozen sections (8 µm) of chronic active multiple sclerosis lesions. Some perivascular infiltrates contain CD200R-positive cells (A), but others do not (B). Sometimes perivascular foamy macrophages express CD200R (C). Bar is 50 µm (A and C) and 100 µm (B).

Discussion

In this study we show that in the human CNS neurons express CD200, likely on the cell membrane and in some neurons also in the cytoplasm, confirming previous publications on the rat CNS and the human retina.[159,160] The cytoplasmic staining that was visible in some neurons may reflect transport of CD200 from the golgi to the cell membrane. It is not clear why this was not seen in the cytoplasm of all neurons, as e.g. that of Purkinje cells was negative, although this might be indicative for differential turnover rates of CD200 on these neurons. We are the first to report that human microglia express CD200R. These data indicate that neurons can suppress microglia via CD200-CD200R interaction to protect themselves from deleterious inflammatory reactions, as previously postulated for rodents. In white matter, CD200 was present on axons. Nevertheless, it is not well understood how axonal CD200 can bind its receptor since the axon is covered with myelin, except for the nodes of Ranvier. For effective immune suppression in white matter, it would therefore be reasonable if CD200 was also expressed by cells surrounding axons. Indeed, CNPase positive oligodendrocytes, but not GFAP positive astrocytes, appeared to express CD200. Hence, these results strongly suggest that not only neuron-glia but also glia-glia interactions are involved in the control of microglia activation.

The expression pattern of CD200 in grey matter is peculiar and difficult to interpret. The staining was diffuse and present on and between neuronal cell bodies. Although the majority of the immunoreactivity is ascribed to neurons, it is possible that oligodendrocytes account for the inter-neuronal staining. It is unlikely that the diffuse staining is caused by secretion of CD200 because it is a membrane-bound glycoprotein encompassing a transmembrane domain, lacking any known proteinase motifs that would be necessary for its release.[164] The diffuse staining pattern cannot be attributed to aspecificity of the antibody, as this was ruled out by specificity tests for this antibody.[120] Furthermore, immunoreactivity was absent in control stainings which included isotype-matched antibodies. Also CD200 expression in the CNS of mice showed a similar pattern as that in the human CNS, but staining was completely absent in $CD200^{-/-}$ mice, confirming specificity of our CD200 staining. Finally, such a diffuse expression pattern as that of CD200 is not uncommon in the CNS as, for example, the synaptic modulatory phosphoprotein GAP43 has been extensively described as a neuron-specific protein, but shows an expression pattern in grey matter areas resembling that of CD200.[165,166]

In contrast to the CNS of control subjects, CD200 was expressed in reactive astrocytes associated with MS lesions. This was a surprising finding as we recently showed that the overall expression levels of CD200 were decreased in the center

and rim of MS lesions.[111] This decreased expression is presumably due to axonal and oligodendrocyte damage, and may enhance macrophage and microglia activation. Since activated macrophages and microglia are thought to drive lesion formation in MS,[42] loss of CD200 will thus enhance inflammation and disease progression, as demonstrated in animal models. The implications for neuronal integrity are clearly reflected in enhanced neuronal damage in these *in vivo* models but also in macrophage-neuronal co-cultures,[125,126,128] whereas animals with increased CD200 levels display enhanced neuroprotection.[129] Our data on CD200-positive reactive astrocytes now suggest that immune activation within the CNS may lead to a protective molecular reflex by astrocytes that are otherwise not expressing CD200. Moreover, it corroborates the idea that astrocytes are protective and neurotrophic.[167] However, overall CD200 expression in MS lesions was still decreased compared to control tissues, indicating that the CNS' response to restore immune suppression in MS fails.

Expression of CD200R on microglia could not be detected using immunohistochemistry, but using flow cytometry we demonstrate that human primary microglia expressed low levels of CD200R, a finding also noted in rodents.[121,136] In contrast to microglia, CD200R was intensely stained on macrophages located in the perivascular space, in close proximity to CD200-void vascular endothelium. Hence, it is likely that in an environment where CD200 is abundantly present, the expression of CD200R is down regulated.

In conclusion, many molecules have been identified to be involved in the activation of macrophages and microglia, such as derived from cellular debris to apoptotic cells. Molecules as CD200 suppress the activation of myeloid cells in order to limit tissue damage, thereby protecting the vulnerable neurons from deleterious effects of inflammation.[168,169] Importantly, also oligodendrocytes and astrocytes can mediate neuroprotection by direct cell-cell contact through CD200-CD200R, in addition to the release of, for example, neurotrophic factors. Dysregulation of the finely tuned resident innate immunity in the CNS may cause macrophage/microglia activation as abundantly occurs in MS and also in other neurodegenerative disorders such as in stroke, Alzheimer's and Parkinson's disease.[170,171] At present, it is not known how expression of CD200 or CD200R is regulated. This will be the next step in providing tools for therapeutic use of immune suppressive mechanisms in the CNS in (inflammatory) neurodegenerative diseases.

Acknowledgments

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4

CD200R expression and regulation on macrophages

Expression of the inhibitory CD200R is associated with alternative macrophage activation

Nathalie Koning, Marco van Eijk, Walter Pouwels, Michael S. M. Brouwer, David Voehringer, Inge Huitinga, Robert M. Hoek, Geert Raes, Jörg Hamann

Submitted

Abstract

The mechanisms by which type II cytokine-associated M2 macrophages maintain their functions are not fully understood. However, signaling pathways known to be important in type I cytokine-associated classically activated M1 macrophages, such as MAPK p38, ERK, and JNK, can be inhibited by signaling through the inhibitory receptor CD200R. Here, we show that CD200R expression was induced on human in vitro-polarized macrophages of the alternatively activated M2a subtype, generated by incubation with IL-4. CD200R induction was restricted to M2-inducing conditions, in contrast to the mannose receptor, which is currently viewed as the most common M2a marker in human. Experimental parasite infections in mice evoke highly polarized type 2 immune responses correlating with an M2 macrophage phenotype. Peritoneal M2a macrophages, elicited during infection with Taenia crassiceps or Trypanosoma b. brucei expressed increased CD200R levels compared to those derived from uninfected mice. However, in vitro stimulation of mouse peritoneal macrophages and T. crassiceps infection in IL-4$^{-/-}$ and IL4R$^{-/-}$ mice showed that, in contrast to human, induction of CD200R in mice was not IL-4 dependent. Our data identify CD200R as a suitable marker for alternatively activated macrophages in human and corroborate observations of distinct mechanisms regulating macrophage polarization in mouse and man. Since the effects of CD200R triggering involve the inhibition of pathways of classical M1 activation, CD200R expression on M2 cells might form a mechanism to keep M2 cells in a polarized state.

Introduction

Macrophages are versatile players in the immune system. They effectively neutralize pathogens and harmful endogenous products via phagocytosis, but also contribute to efficient immunity by production and secretion of multiple mediators like cytokines and chemokines. Next to pro-inflammatory activities, macrophages possess important regulatory activities during the resolution phase of an immune response and at sites where antigenic material is ignored (lung and colon) or tolerated (eye, brain, ovary, and testis). Opposing macrophage activities result from the initiation of distinct gene expression programs.[23,172-174] IFN-γ and LPS induce classic activation of macrophages. These macrophages, also known as M1 cells, secrete high amounts of pro-inflammatory cytokines and reactive oxygen species and efficiently clear pathogens either directly or by facilitating Th1-mediated responses. In contrast, stimulation with IL-4, IL-10, IL-13, or glucocorticoids triggers pathways that program macrophages to exert anti-inflammatory functions. These type 2 cytokine-associated M2 cells can be further classified into M2a, M2b, and M2c cells that each emphasize specific functions.[23] For example, IL-4 generates M2a cells that are considered to be involved in Th2-mediated responses and are most commonly characterized by mannose receptor (MR, CD206) expression.[172] IL-10 and glucocorticoids lead to M2c-polarized cells that mediate tissue remodeling and matrix deposition and are characterized by high levels of the scavenger receptor CD163.[175]

The way M2 cells maintain their immune suppressive properties has not been completely elucidated. However, immune inhibitory receptors such as the membrane glycoprotein CD200R may be involved for several reasons. CD200R, mainly expressed on myeloid cells and on a subset of T and B cells,[121,122,125] inhibits inflammatory macrophage activation as illustrated by studies showing that absence of its ligand CD200 aggravates, accelerates, or increases the susceptibility of inflammatory models such as experimental autoimmune encephalomyelitis, uveoretinitis, and collagen-induced arthritis.[125,126] Indeed, CD200R signaling profoundly inhibits the MAP kinases p38, ERK, and JNK, the common signaling pathways involved in classical activation of macrophages.[123] As M2 cells can mediate a type II immune response, by producing and secreting anti-inflammatory mediators, and reducing inflammatory cytokines like TNF, it is interesting that some of these effects have also been reported for CD200R.[124] However, the expression of CD200R or other immune inhibitory molecules on M2 cells has never been studied thoroughly.

Methods

Cell culture

Monocytes were purified from human peripheral blood mononuclear cells and either matured into macrophages for 4 days and then polarized for 3 days with IFN-γ (50 ng/ml), IL-1β (40 ng/ml), TNF (50 ng/ml), GM-CSF (50 ng/ml), M-CSF (25 ng/ml), IL-4 (40 ng/ml), IL-13 (50 ng/ml), IL-10 (50 ng/ml) (all Peprotech, London, UK), or dexamethasone (2 μM, Sigma Aldrich, Zwijndrecht, The Netherlands), or were directly matured and polarized for 3 days in the presence of these stimuli, as described previously.[176] Mouse peritoneal macrophages were isolated as described previously[177] and were stimulated with 50 ng/ml mIL-4 (R&D Systems, Minneapolis, MN) or 50 ng/ml mIL-10 (Peprotech) for 3 days.

Quantitative PCR

Total RNA (10 ng per reaction) was transcribed into first-strand cDNA and analyzed by quantitative PCR (qPCR) using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) and the ABI Prism 7300 Sequence Detection System (Applied Biosystems) as described elsewhere.[111] Target genes were human CD200R (forward primer 5′-GAGCAATGGCACAGTGACTGTT-3′, reverse primer 5′-GTGGCAGGTCACGGTAGACA-3′), MR (forward primer 5′-TGCAGAAGCAAACCAAACCTGTAA-3′, reverse primer 5′-CAGGCCTTAAGCCAACGAAACT-3′), CD163 (forward primer 5′-ACATAGATCATGCATCTGTCATTTG-3′, reverse primer 5′-ATTCTCCTTGGAATCTCACTTCTA-3′), TREM2 (forward primer 5′-CCACCCACTTCCATCCTTCT-3′, reverse primer 5′-GTCCCTGGCTTCTGTCCAT-3′), and SIRPα (forward primer 5′-GTCTGGAGCAGGCACTGA-3′, reverse primer 5′-GGACTCGCAGGTGAAGCT-3′) and mouse CD200R (forward primer 5′-TGCCATCCTGCACAATAGCA-3′, reverse primer 5′GGAGGCCCAGGTGATGTT-3′). Targets were normalized to 18S ribosomal RNA (forward primer 5′-TAGTCGCCGTGCCTACCAT-3′, reverse primer 5′-CCTGCTGCCTTCCTTGGA-3′).

Flow cytometry

Cells were incubated with anti-human Alexa 674-labeled CD200R (Serotec, Oxford, UK), APC-labeled MR (BD Biosciences, San Jose, CA), PE-labeled CD163 (BD Biosiences), and biotinylated TREM2 (Peprotech) antibodies. Appropriate Ig isotypes were used as negative control. Staining was measured on a FACSCalibur and analyzed with FlowJo software version 8.7.1 (Treestar, Inc. Ashland, OR).

Parasite infections

Inoculation of C57BL/6 and Balb/c mice with *Taenia crassiceps* and of C57BL/6 x BALB/c (F1) mice with PLC$^{-/-}$ *Trypanosoma b. brucei* has been described previously.[177]

Statistical analysis

Data were analyzed by one-way ANOVA and post-hoc Dunnett's Multiple Comparison Test using GraphPad Prism 5.01 (GraphPad Software Inc. La Jolla, CA, USA). A p value < 0.05 was considered significant.

Results and Discussion

To determine the expression of the immune inhibitory receptor CD200R on polarized macrophages, we subjected human monocytes, purified from peripheral blood mononuclear cells (PBMCs), to a panel of pro- and anti-inflammatory stimuli for 3 days, as described previously,[176] to initiate either M1 activation (INF-γ, IL-1β, TNF, GM-CSF) or M2 activation (IL-4, IL-13, IL-10, M-CSF or dexamethasone) (Fig. 1).[178] Analysis of CD200R mRNA expression in these cells, performed by quantitative real-time PCR (qPCR), revealed a significant induction after stimulation with IL-4 or IL-13 (Fig. 1). On the protein level, CD200R expression was robustly increased by IL-4 stimulation and slightly but not significantly following IL-13, IL-10, and the glucocorticoid dexamethasone (Fig. 1). These results indicate that CD200R is solely expressed on alternatively activated M2a macrophages, which are at present most commonly identified by expression of the MR.[172] We therefore compared the expression pattern of CD200R with that of MR mRNA by qPCR and protein by flow cytometry in the same series of experiments. MR mRNA was indeed induced by IL-4 and IL-13, but also by the M1 activator GM-CSF, confirming previously reported data,[179] and to some extent by IL-1β, although the latter was not significant (Fig. 1). Different from M2a cells are M2c cells that are induced by IL-10 and glucocorticoids and are characterized by CD163 expression.[23] As expected, CD163 mRNA and protein was induced only by IL-10 and the glucocorticoid dexamethasone and not by IL-4 or IL-13 (Fig. 1), confirming the M2c specificity of this marker. Thus, whereas CD163 is an excellent marker for M2c cells, CD200R is expressed on M2a cells with higher specificity than MR and therefore appears to be a novel reliable marker for this type of macrophage *in vitro*.

We next tested whether two other inhibitory molecules expressed on myeloid cells, TREM2 and SIRPα,[113,137,180] were also primarily expressed by alternatively acti-

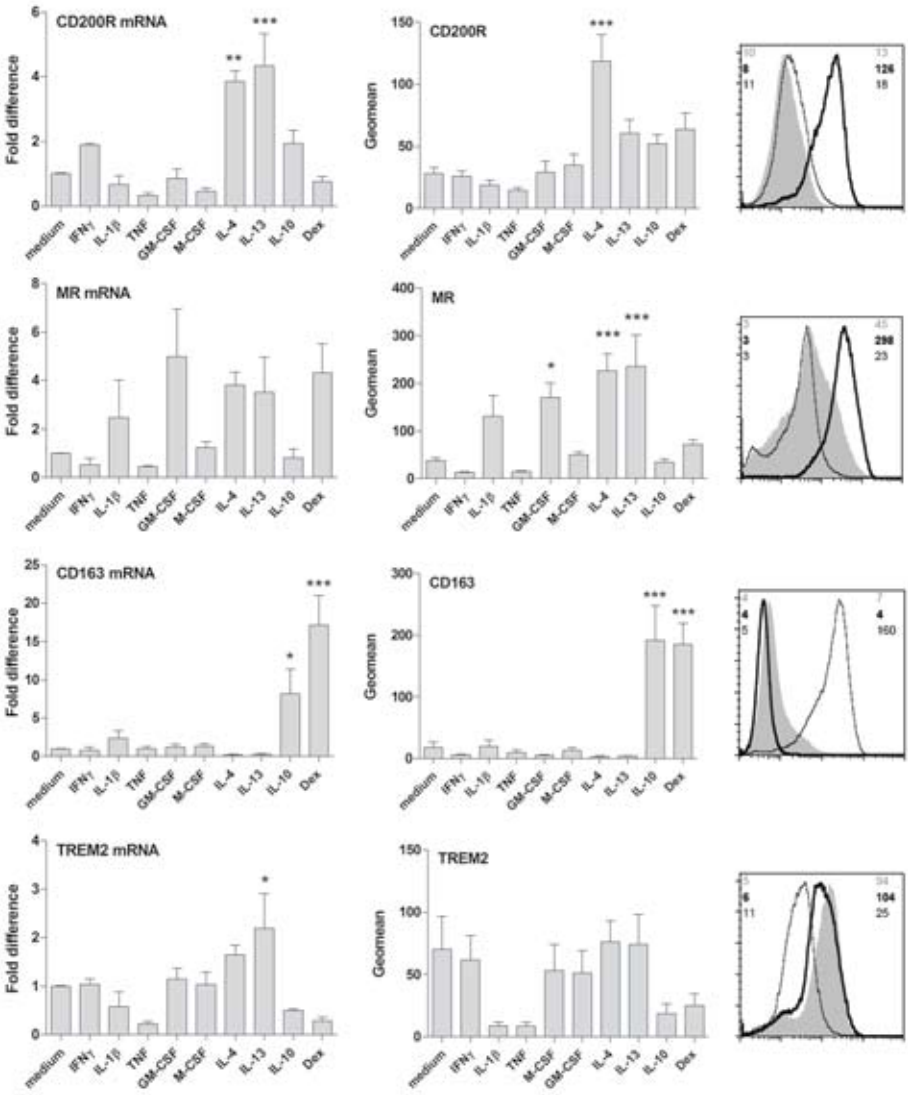

Fig. 1 Expression of CD200R by human macrophages maturated under polarizing conditions. Human primary monocytes were isolated from PBMCs and differentiated into M1 macrophages by stimulation with IFN-γ, IL-1β, TNF, or GM-CSF or into M2 macrophages by stimulation with M-CSF, IL-4, IL-13, IL-10, or dexamethasone. After 72 hours, mRNA and protein expression of CD200R, MR, CD163, and TREM2 was determined. Transcript data (left panels) are depicted as mean-fold induction ± SEM compared to macrophages cultured in medium only (n = 3). Protein data (middle panels) are depicted as mean fluorescence intensity (geomean) ± SEM (CD200R, n = 12; MR, n = 5; CD163, n = 6; TREM2, n = 3). Representative histograms (right panels) show protein expression levels of macrophages stimulated with IL-4 (bold line), IL-10 (thin line), or unstimulated (medium, grey) with geometric means indicated in the numbers with corresponding colors (isotype controls left and antibodies right). Single asterisk indicates $0.01 < p \leq 0.05$; double asterisks indicate $0.001 < p \leq 0.01$; triple asterisks indicate $p < 0.001$.

vated macrophages. A significant induction of TREM2 was found after IL-13 stimulation on mRNA level, but not on protein level (Fig. 1). IL-4 did not induce TREM2 expression. Expression of SIRPα, determined at the mRNA level, was not induced by any of the investigated stimuli (data not shown), indicating that induction of immune inhibitory molecules is not a common phenotype of experimentally polarized human M2 macrophages, and seems restricted to CD200R.

To further analyze the induction of CD200R expression on M2 cells, freshly isolated monocytes from PMBCs were first matured for 4 days and then stimulated with IL-4 or IL-13 for another 3 days. Figure 2 shows that this procedure induced a dramatic increase in CD200R mRNA expression (Fig. 2) compared to cells that were stimulated directly after isolation (Fig. 1). Similar to CD200R, also the increase in MR expression was much stronger when cells were matured prior to stimulation (Fig. 2). As expected, expression of CD163 was unaffected by IL-4 or IL-13 (Fig. 2).

Experimental infection of mice with parasites is known to provoke strong type 2 immune responses correlating with an M2 signature of the elicited macrophages[177] To test whether CD200R is expressed on M2 cells *in vivo*, we analyzed peritoneal macrophages isolated from animals infected with either the helminth *Taenia crassiceps* or the protozoan *Trypanosoma b. brucei*, inoculated as described previously.[177] Indeed, macrophages from T. crassiceps-infected mice (C57BL/6 and BALB/c) showed increased CD200R mRNA expression compared to those derived from uninfected mice (Fig. 3A). Mice infected with phospholipase C-deficient ($PLC^{-/-}$) *T. b. brucei* display a switch from a type 1 cytokine milieu during the early stage of the

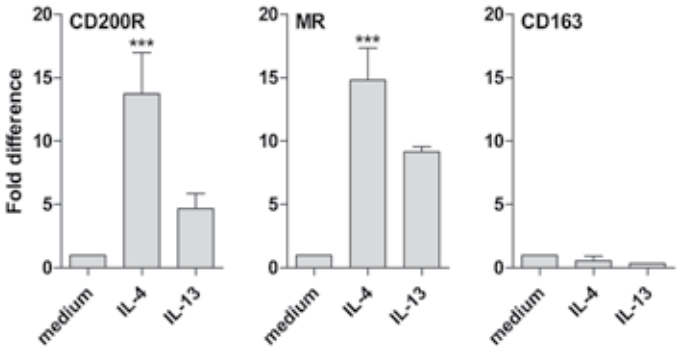

Fig. 2 Expression of CD200R by maturated human macrophages that subsequently were polarized into M2 cells. Human primary monocytes were isolated from PBMCs and adhered to the culture dish for 4 days to obtain matured macrophages, and were then differentiated with IL-4 or IL-13. After 3 days, mRNA expression of CD200R, MR and CD163 was determined by qPCR. The mRNA data are depicted as mean-fold induction ± SEM as compared to macrophages cultured in medium only (n = 4-6). Triple asterisks indicate $p < 0.001$.

infection to a type 2 environment in the chronic phase of the disease.[177] CD200R expression was unaltered or even slightly decreased on peritoneal macrophages derived from the acute phase, but robustly up-regulated on macrophages derived from the chronic phase (Fig. 3A). In both parasite models, regulation of CD200R expression correlated well with the established mouse M2 markers arginase-1 and 'found in inflammatory zone 1' (Fizz1)[177] (data not shown). These results indicate that CD200R is also induced on mouse M2 macrophages *in vivo*.

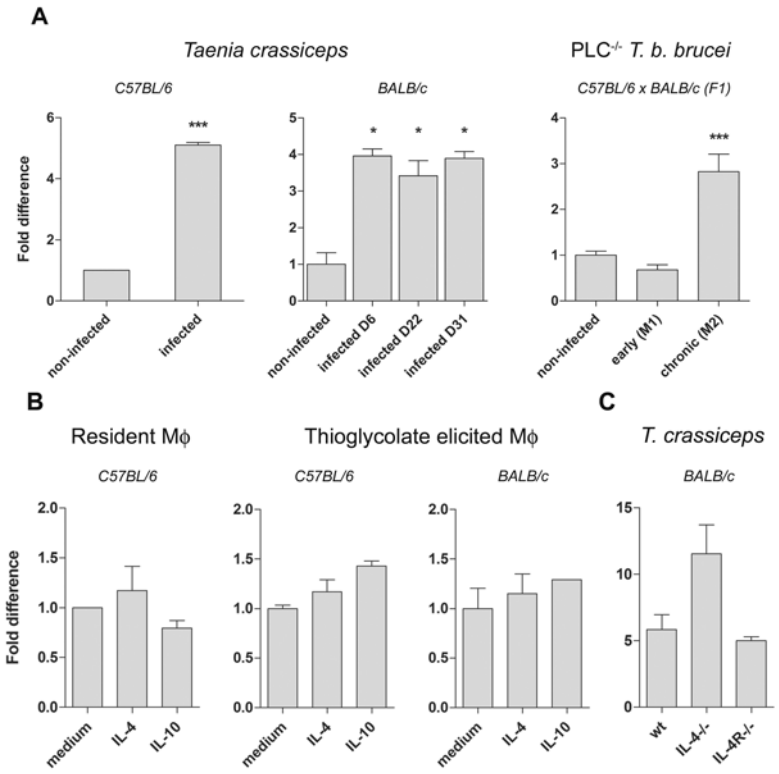

Fig. 3 CD200R expression in mouse alternatively activated macrophages *in vivo* and *in vitro*. (A) Transcription of CD200R was determined in peritoneal macrophages isolated from C57BL/6 mice at 4 weeks after infection with *Taenia crassiceps* (left panel), and from BALB/c mice at 6, 22, and 31 days after infection (central panel). CD200R expression was also determined in peritoneal macrophages isolated from C57BL/6 x BALB/c (F1) mice in the early (2 weeks) and chronic (3-4 months) stage of infection with PLC$^{-/-}$ *Trypanosoma b. brucei* (right panel). (B) Induction in CD200R mRNA in resident peritoneal macrophages (Mφ) isolated from C57BL/6 mice and thioglycolate-elicited macrophages isolated from C57BL/6 and BALB/c mice, stimulated with IL-4 or IL-10. (C) Induction of CD200R mRNA on peritoneal macrophages isolated from BALB/c wild type, IL-4$^{-/-}$ and IL-4R$^{-/-}$ mice infected with *T. crassiceps*. Data are depicted as mean-fold induction ± SEM compared to macrophages from non-infected mice (A), untreated cultures (B), and non-infected wild-type mice (C) (n = 2-6). Single asterisk indicates $0.01 < p \leq 0.05$; triple asterisk indicates $p < 0.001$.

We next determined whether CD200R expression on M2 macrophages in mice was IL-4 dependent, as we showed in human macrophages. Surprisingly, mIL-4 or mIL-10 failed to induce CD200R mRNA expression in resident peritoneal macrophages from C57BL/6 mice, and in thioglycolate-elicited peritoneal macrophages from either C57BL/6 (Th1-prone) or BALB/c (Th2-prone) mice (Fig. 3B). *In vivo* we confirmed that the elevated CD200R expression in M2 cells was indeed IL-4 independent as no abrogation of CD200R induction was observed in IL-4$^{-/-}$ or IL-4R$^{-/-}$ mice infected with *T. crassiceps* (Fig. 3C).

In summary, we here demonstrate that the immune inhibitory molecule CD200R is induced on *in vitro*-polarized human macrophages following IL-4 stimulation and can be used as a novel cell surface marker for M2a cells in human, with even higher specificity than MR. Furthermore, CD200R is also expressed on mouse M2 cells, but in contrast to human, is not induced by IL-4. In fact, it was recently shown that IFN-γ was able to induce CD200R expression in mouse peritoneal macrophages.[181] Since we show that IFN-γ does not affect CD200R expression in human macrophages, these data corroborate previous findings implying differences in human versus mouse macrophage biology.[177,182] The current classification in macrophage polarization represents extremes of a continuum, indicating that CD200R may also be expressed on macrophages with phenotypes lying in between these extremes. However, the present data imply that rather than merely cellular silencing, it is now more likely that CD200R may be actively involved in maintaining M2 cells in their polarized state, which enables them to create a milieu that favors a type 2 immune response. Lack of this regulatory pathway, for instance in CD200$^{-/-}$ mice, could then lead to exacerbation of (auto)immune diseases as reported previously.[125,126,181]

Acknowledgments

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5

Isolation and culture of human post-mortem resident microglia

A pilot study of CD200 and CD200R regulation in the human CNS

Nathalie Koning, Jeroen Melief, Marco van Eijk, Alina Glowacki,
Jörg Hamann, Inge Huitinga, Robert M. Hoek

Abstract

Studies of human microglia are exceptional as these cells are difficult to access. It is however, very important to study microglia biology, because they have been implicated in many neurodegenerative conditions. In the present study we show a rapid procedure to isolate, sort and culture microglia with high purity from human post-mortem brain tissue. The techniques that are used are based on density gradients and cell surface expression of CD11b and CD45, as elucidated in cell populations derived from human blood, choroid plexus and corpus callosum white matter. Because in vitro stimulation of monocyte-derived macrophages results in polarized cells with different pro- and anti-inflammatory properties, we tested whether microglia, the resident macrophages of the brain, may polarize as well. We therefore stimulated cultured primary microglia with IL-4, as we recently demonstrated that IL-4 induced expression of the immune inhibitory molecule CD200R and mannose receptor (MR) in human alternatively activated macrophages. Although microglia expressed CD200R, expression was not up-regulated by IL-4. In addition, MR was not detectable in microglia, independent of IL-4 stimulation. This was in contrast to choroid plexus macrophages where IL-4 increased both CD200R and MR expression. Thus, MR may be the first discriminating molecule between human macrophages and microglia. Since CD200 provides an important mechanism to control microglia activation via CD200R, we additionally addressed CD200 expression on a human neuroblastoma cell line. IFN-γ, IL-1β, TNF, IL-4, IL-10 or dexamethasone did not influence CD200 mRNA expression. Conclusively, these data demonstrate that microglia behavior differs from that of other tissue macrophages. Furthermore, the expression of CD200R on human microglia and CD200 on neurons is confirmed but their regulation remains unidentified. Most importantly, the currently described techniques offer many possibilities to study the biology and behavior of human microglia directly ex vivo.

Introduction

Microglia originate from myeloid progenitor cells and are the predominant type of immune cells in the brain. They closely resemble macrophages, and share surface markers like CD11b and functions, such as their phagocytic capacity. Depending on the stimulus, macrophages can turn on so-called classical or alternative activation programs. Alternative macrophage polarization results in a group of divergent cells named M2a, M2b and M2c, that emphasize the different anti-inflammatory properties such as tissue repair and remodeling, scavenging of debris and dampening inflammation by promoting type II immune responses.[22,23,172-174] Whether microglia can polarize is at present unknown, and is an important topic of investigation as there are many controversies about the contribution of activated microglia in many neurodegenerative diseases. For example, it has been proposed that a disproportionate activation of microglia leads to an uncontrolled (inflammatory) reaction in neurodegenerative diseases.[112] On the other hand, several studies suggest a protective role for microglia, implying that also microglia activation may be heterogeneous resulting in different outcomes.[31]

Microglia activation is tightly regulated amongst others by the inhibitory molecule CD200 receptor (CD200R). CD200R is present on all myeloid cells where it has been shown to be a potent immune suppressor by dampening immunological activity via inhibition of MAPK pathways.[121,123,124] Although microglia express only low levels of CD200R,[121,136,183] absence of its ligand CD200, expressed by neurons and oligodendrocytes, is sufficient for spontaneous activation of microglia.[125] It is known that IL-4 induces alternatively activated monocyte-derived macrophages of the M2a subtype, which are involved in anti-inflammatory type II responses. We recently demonstrated that CD200R expression is enhanced by IL-4 in these cells, with even higher specificity than mannose receptor (MR), the currently most common M2a marker, since MR was also induced by GM-CSF (Koning *et al.* submitted). We therefore hypothesized that IL-4 stimulation could also induce alternative activation in microglia, the resident macrophages of the brain, and wondered whether this would also be reflected by increased CD200R expression levels. In order to address this question, we needed to develop a method to isolate and culture human microglia.

The biology of human primary microglia in a pathological setting, such as multiple sclerosis (MS), is a largely unexplored field, primarily because these cells are poorly accessible, and brain tissue from MS patients is scarce. It is important that techniques are developed that allow the studying of primary microglia. The currently most frequent used technique to isolate microglia is based on adhesive prop-

erties and is derived from neonatal rodent brains. There are many disadvantages of this technique, for example the source of the material, the time that is needed to obtain the cells and the purity of the cultures. A more reliable method to obtain pure microglia would be to use fluorescence activated cell sorting (FACS). Pioneers in this field are Sedgwick and colleagues, who showed the characterization and isolation of rodent, and later also human resident microglia using flow cytometric sorting based on expression profiles of CD11b and CD45.[162,184,185] Based on these experiments, we show in the present study a functional procedure to isolate, sort and culture pure resident microglia from post-mortem human brain tissue. In a pilot study, we further show that unlike macrophages, IL-4 does not induce CD200R or MR expression in these cells. Furthermore, since its ligand CD200 is highly expressed on neurons and is essential for controlling microglia activity via CD200R,[111,125] we studied the regulation of CD200 on a human neuroblastoma cell line in parallel.

Methods

Human microglia isolation

All material has been collected from donors from whom a written informed consent for brain autopsy and the use of the tissue and clinical information for research purposes had been obtained by the Netherlands Brain Bank (NBB). Corpus callosum white matter (4 grams) and choroid plexus, provided by the NBB, was obtained at autopsy and stored in Hibernate A medium (Brain Bits LLC, Springfield, IL) at 4°C. Tissue was obtained from 8 male and 6 female donors diagnosed with Alzheimer's disease (4 donors), Pick's disease (2 donors), Lewy Body disease (2 donors), Parkinson's disease (1 donor), epilepsy (1 donor), bipolar disorder (1 donor), progressive supranuclear palsy (1 donor) and without neurological condition (2 donors). The average age of the donors was 78 years (range 50 – 93 years). The average post-mortem delay was 5 hours and 48 minutes (range 4 hours and 10 minutes – 9 hours). Within 3 – 18 hours after autopsy, cells were isolated using a Percoll gradient, as described previously,[162] with some adaptations. In brief, tissue (per 2 grams) was mechanically dissociated using a metal sieve in a total volume of 50 ml GKN/0.2% BSA (8 g/l NaCl, 0.4 g/l KCl, 1.77 g/l $Na_2HPO_4.2H_2O$, 0.69 g/l $NaH_2PO_4.H_2O$, 2 g/l D-(+)-glucose, 2 g/l BSA, pH 7.4). After centrifugation (400 x g, 7 min), the pellet was reconstituted in 5 ml dissociation buffer (4 g/l $MgCl_2$, 2.55 g/l $CaCl_2$, 3.73 g/l KCl, 8.95 g/l NaCl, pH 6-7) and enzymatically digested in 150 U Collagenase Type I (Worthington, Lakewood, NJ) and 200 µg/ml DNase I (Roche Diagnostics, Mannheim, Germany) for 1 h at 37°C. During this incubation, the tissue was mechanically dis-

rupted by passing the mixture through a p1000 pipet tip with decreasing bore size every 5-10 minutes. After washing in GKN/0.2% BSA, the cells were resuspended in 20 ml Percoll (ρ = 1.03), underlain by 10 ml Percoll (ρ = 1.095) and overlain by 5 ml GKN/0.2%BSA and centrifuged at 1200 x g for 30 min with slow acceleration and no break. The myelin layer was removed from the GKN-Percoll 1.03 interface and discarded, after which the cells from the 1.03-1.095 interface were collected. The average number of recovered cells was $3x10^6$ cells/gram tissue.

Fluorescent activated cell sorting

The freshly isolated cells from corpus callosum, choroid plexus or human blood, were incubated with phycoerythyrin (PE)-labeled CD11b (Clone ICRF44, DakoCytomation, Glostrup, Denmark), fluorescein isothiocyanate (FITC)-labeled antibody against human CD45 (Clone HI30, Dako) or with isotype-matched controls in the presence of 10% human pool serum (HPS) for 1 h at 4°C. Antibody dilutions and washing steps were performed in FACS buffer (PBS, 0.2% BSA, 10 mM sodium azide). After washing, the cells were analyzed using a FACSCalibur flow cytometer (BD PharMingen, San Jose, CA) and the data was analyzed using FlowJo software version 8.7.1 (Treestar, Inc. Ashland, OR). To isolate microglia from the CNS, cells were stained with PE-labeled CD11b in the presence of 10% HPS for 1 h at 4°C. Antibody dilutions and washing steps were performed in GKN/0.3%BSA. After washing, the $CD11b^+$ cells were sorted on a FACS Aria cell sorter (BD Biosciences).

Cell culture

Isolated primary cells obtained after sorting were cultured in 96-wells flat bottom culture plates in RPMI (Gibco, Invitrogen, Breda, The Netherlands) with 1% heat-inactivated HPS, and were stimulated with 40 ng/ml human IL-4 (Peprotech, London, UK) for 72 hours. The human neuroblastoma cell line SH-SY5Y (kindly provided by dr. E. Hol, Netherlands Institute for Neuroscience, Amsterdam) was cultured in 24-wells in serum-free Neurobasal medium (Invitrogen, Carlsbad, CA, USA), supplemented with the neuronal growth factor B-27 (Invitrogen). Cells were stimulated with $1x10^4$ nM dexamethasone (Sigma Aldrich, Zwijndrecht, the Netherlands), 50 ng/ml IL-1β, 5 ng/ml IL-4, 20 ng/ml IL-10, 10 ng/ml TNF or 50 ng/ml IFN-γ (Peprotech) for 6 and 24 hours.

RNA isolation

The cells were washed in PBS and lysed in 1 ml (SH-SY5Y cells) or 800 µl (primary cells) TRIZOL (Invitrogen). Respectively 200 or 160 µl chloroform (Sigma) was added after which the lysate was thoroughly mixed and centrifuged at 12,000 rpm, 4°C for

15 minutes to recover the aqueous phase. RNA from SH-SY5Y cell lysates was precipitated with isopropyl alcohol, dissolved in 30 µl RNAse free water and stored at -20°C until further use. For the primary cell lysates, an equal volume of 70% ethanol was added to the collected aqueous phase, and loaded onto an RNeasy Mini Kit column (Qiagen, Hilden, Germany). Further RNA isolation was performed according to the RNeasy protocol (RNeasy Mini Handbook 06/2001). The amount (OD260) and purity (OD260/280 ratio) of the RNA was determined using a nanodrop (ND-1000, NanoDrop Technologies, Rockland, DE, USA).

cDNA synthesis and quantitative PCR

The synthesis of cDNA was performed as described previously.[111] Real-time quantitative PCR (qPCR) was performed with SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) with samples containing equal cDNA concentrations of 10 ng initial total RNA per reaction. Analysis was performed according to manufacturer's protocol and the ABI Prism 7300 Sequence Detection System (Applied Biosystems). Expression of CD200 (fw: 5'-CCAGGAAGCCCTCATTGTGA-3', rv: 5'-TCTCGCTGAAGGTGACCATGT-3'), CD200R (fw: 5'-GAGCAATGGCACAGTGACTGTT-3', rv: 5'-GTGGCAGGTCACGGTAGACA-3'), MR (fw: 5'-TGCAGAAGCAAACCAAACCT-3', rv: 5'-CAGGCCTTAAGCCAACGAAACT-3') and HLA-DR (fw: 5'-CCCAGGGAAGACCACCTTT-3', rv: 5'-CACCCTGCAGTCGTAAACGT-3') were normalized to 18S ribosomal RNA. For each primer pair, the primer efficiency (Effpr) was calculated using LinRegPCR software.[145] Fold differences were calculated by $(Effpr)^{-\Delta\Delta CT}$ method.[146]

Results

Isolation and sorting of human primary microglia based on CD11b/CD45 expression

Reliable isolation and culture of human microglia facilitates the study of gene or protein regulation in these cells. Currently used methods to isolate microglia from brain homogenates based on adhesion are time-consuming and may be contaminated with other CNS cell types or blood-derived myeloid cells. A more reliable way of isolating microglia would be to sort these cells based on a cell surface marker, but the lack of a specific microglia marker impedes the discrimination of microglia from CNS associated macrophages or monocyte-derived macrophages. Successful microglia isolation in rodents was established based on the staining for CD11b (myeloid cells) and CD45 (leukocytes) cell surface markers, as macrophages are $CD11b^{+}CD45^{hi}$ while microglia are $CD11b^{+}CD45^{dim}$.[162,184,185] To analyze whether this feature would come to our aid also in the isolation of human primary microglia, we

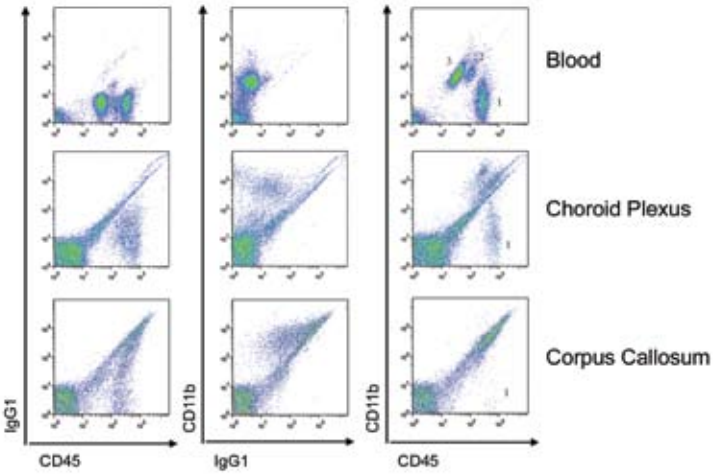

Fig. 1 Flow cytometric analysis of CD11b and CD45 on cells isolated from peripheral blood, choroid plexus and corpus callosum. The left and middle panel show single CD11b$^+$ and CD45$^+$ labeled populations in the three tissues. In choroid plexus and corpus callosum, autofluorescent cell populations are evident. The left panel shows double labeling for CD11b and CD45. In blood, the double labeling shows three populations: lymphocytes (population 1), granulocytes (population 2) and granulocytes (population 3). Population 1 is also present in cells isolated from choroid plexus, but contained very few events when derived from corpus callosum. The CD11b$^+$CD45$^+$ cells from choroid plexus may contain both macrophages and granulocytes. The CD11b$^+$CD45$^+$ population derived from corpus callosum lies underneath the autofluorescent cells. Representative data of 3 (blood and choroid plexus) and 10 (corpus callosum) experiments are shown.

set out to isolate cells from peripheral blood, choroid plexus and corpus callosum. Blood-derived cells showed two CD45$^+$ populations (Fig. 1, left panel) and a single CD11b$^+$ population (Fig. 1, middle panel). These leukocyte populations were separated by double labeling for CD45 and CD11b (Fig. 1, right panel) into lymphocytes (population 1), granulocytes (population 2) and monocytes (population 3). Cell isolates from choroid plexus, a well vascularized site lining the ventricles and highly enriched for macrophages, contained a single CD11b$^+$ and CD45$^+$ population as well as an autofluorescent cell population (Fig. 1). By double labeling for CD11b and CD45, the lymphocyte population was identified (population 1). The CD11b$^+$CD45$^+$ population consisted mostly of monocytes/macrophages, but probably also contained granulocytes. However, the discrimination between these cell types was hampered by interference of autofluorescent events. Similar to choroid plexus, cells derived from corpus callosum contained a large autofluorescent population (Fig. 1). Whereas single staining clearly identified CD45$^+$ and CD11b$^+$ populations, double labeling resulted in only one double positive population, which largely overlaps with the autofluorescent population. This is in contrast to CNS myeloid cells from

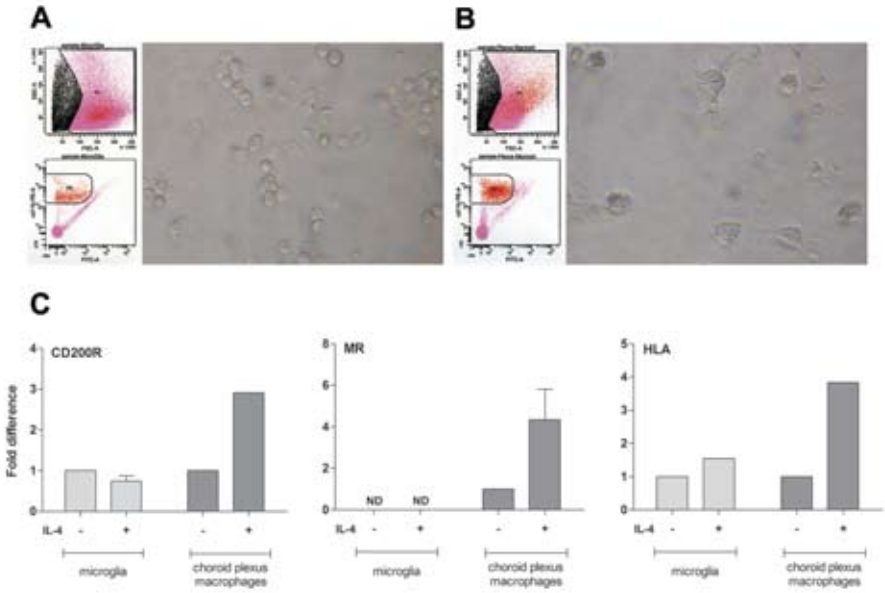

Fig. 2 Morphology of and gene expression in microglia and choroid plexus macrophages sorted based on CD11b expression. (A) Microglia are small cells with little granularity as seen in the forward side scatter analysis (red dots in upper FACS plot). In culture, microglia are small, round cells without ramifications. (B) Choroid plexus macrophages are much larger and more granular as seen in both the forward side scatter analysis (red dots in upper panel) and in culture, where some cells also displayed extensions. (C) After 72 hours of IL-4 stimulation, gene expression of CD200R, MR and HLA-DR was determined and compared to unstimulated cells. Expression of MR was not detectable (ND) in microglia. Results of one or two experiments are shown, and where applicable, mean ± SD are indicated.

rodent brain tissue that comprise of the $CD11b^{+}CD45^{hi}$ and $CD11b^{+}CD45^{dim}$ populations as mentioned before.[162] Moreover, the merger of the double positive population with the autofluorescent events, made it impossible to sort these cells on the basis of differential CD45 staining. In addition, these stainings show hardly any $CD11b^{+}CD45^{hi}$ cells, indicating that nearly all CD11b expressing cells are microglia. Therefore, we chose to sort and culture the $CD11b^{+}$ cells. Also note that there were very few $CD11b^{-}CD45^{+}$ cells (population 1) in corpus callosum, indicating that the fraction of lymphocytes was scarce in this tissue. Since lymphocytes are most likely derived from the vasculature, and are rare in the CNS parenchyma, this suggests that the contamination of blood-derived leukocytes, including monocytes/macrophages, was very low.

IL-4 activation does not induce CD200R expression in human primary microglia

Up to 135,000 $CD11b^{+}$ cells derived from corpus callosum (microglia) and up to 210,000 $CD11b^{+}$ cells from choroid plexus (macrophages) were sorted and cultured

(Fig 2A,B). Both cell types adhered to the culture dish within 60 minutes. Primary microglia in culture displayed a small and round morphology (Fig. 2A), whereas choroid plexus macrophages were much larger, showed several extensions and had a granular appearance (Fig. 2B). We previously showed that IL-4 induced alternative activation of macrophages of the M2a subtype is associated with enhanced CD200R expression (Koning *et al*. submitted). Because microglia originate from myeloid progenitor cells and are commonly called 'the resident macrophages of the brain', we studied this feature in primary microglia and choroid plexus macrophages. Although CD200R mRNA was clearly present in microglia, culturing them for 72 hours in the presence of IL-4 did not alter its expression levels (Fig. 2C). In contrast, IL-4 treatment during this culture period did induce CD200R expression 2.9 times in choroid plexus macrophages isolated from the same donor. Currently the most common marker for M2a cells is the mannose receptor (MR) that is also induced upon IL-4 stimulation in blood-derived macrophages.[172] However, MR mRNA was not detectable in microglia either in the absence or presence of IL-4 (Fig. 2C). In contrast, MR expression in choroid plexus macrophages was present in unstimulated cells, and increased 3.9-fold upon IL-4 stimulation. To confirm whether IL-4 could activate both cell types, HLA-DR expression was determined. HLA-DR was expressed on both cell types with absolute expression levels in microglia resembling that of unstimulated choroid plexus macrophages (data not shown). IL-4 slightly increased HLA-DR expression in microglia but more robustly induced expression in choroid plexus macrophages by 3.8 times (Fig. 2C). Collectively, these data indicate that choroid plexus macrophages resemble blood-derived macrophages, but microglia do not.

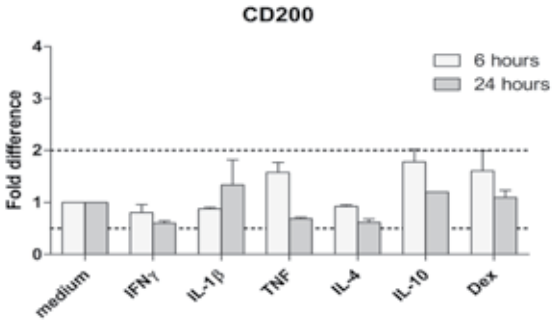

Fig. 3 Regulation of CD200 on neuroblastoma cells. Expression of CD200 mRNA was determined in the neuroblastoma cell line SH-SY5Y upon stimulation with IFN-γ (50 ng/ml), IL-1β (50 ng/ml), TNF (10 ng/ml), IL-4 (5 ng/ml), IL-10 (20 ng/ml) or dexamethasone (Dex, $1x10^4$ nM) for 6 and 24 hours. Dotted lines at 2 and 0.5 correspond to a 2 and -2-fold difference and indicate the sensitivity of the technique, i.e. a difference of at least one cycle threshold. Data represent the mean ± SEM from at least 2 experiments.

CD200 expression is stable on human neuroblastoma cells

To establish whether CD200 expression can be altered, we applied a panel of pro- and anti-inflammatory stimuli to the human neuroblastoma cell line SH-SY5Y. As shown in Figure 3, none of the stimuli convincingly induced or reduced CD200 mRNA expression in these cells, neither after 6 nor after 24 hours. Varying the concentrations of the applied stimuli had no effect either and stimulating the cells for up to 48 hours resulted in reduced viability of the cells (data not shown). These results indicate that CD200 expression on neuroblastoma cells is stable and hardly influenced by cytokines or dexamethasone.

Discussion

In the present pilot study, we show a successful and rapid procedure to isolate and culture human microglia using FACS techniques. Although the isolation procedure of using a density gradient combined with flow cytometry was based on techniques described for rodents by Sedgwick *et al*.[162] and for human biopsy material by Dick *et al*.,[185] the method in the present study using human post-mortem brain tissue from which microglia are sorted and cultured within a few hours after the donor's death is unique and not described elsewhere. This fast procedure allows rapid microglia manipulation in culture as well as gene and protein expression analyses.

Although no specific microglia marker is known, Sedgwick *et al*. used CD11b and CD45 staining intensity to discriminate macrophages that are $CD11b^{+}CD45^{hi}$ from microglia that are $CD11b^{+}CD45^{dim}$.[162] In the present study however, we only detected a single $CD11b^{+}CD45^{+}$ cell population. In the studies of Sedgwick *et al*. and Ford *et al*., a clear $CD11b^{-}CD45^{+}$ lymphocyte population was present in their isolates. As whole rodent brain homogenates were used, these lymphocytes were most likely derived from meninges, subarachnoid space and choroid plexus. Using white matter from corpus callosum that obviously lack these structures, we detected only few lymphocytes, confirming a previously reported observation in human biopsy brain tissue.[185] The contribution of blood-derived $CD11b^{+}CD45^{hi}$ myeloid cells is therefore expected to be low. However, it is still surprising that we could not distinguish between a $CD11b^{+}CD45^{dim}$ and $CD11b^{+}CD45^{hi}$ population in post-mortem corpus callosum as this was previously shown in isolates from human biopsy tissue, although the latter population was remarkably small.[185] It is therefore most likely that blood-derived or perivascular macrophages are not present in our isolates, for example because they actively migrated out of the tissue in the intervening time between isolation and processing of the tissue or they underwent apoptosis.

The most frequently applied technique to obtain microglia, predominantly from neonatal rodent CNS tissue, is based on adhesive properties of microglia after vigorously agitating a mixed cell culture containing amongst others astrocytes, microglia and neurons, for many hours or days with or without GM-CSF or M-CSF.[186-188] The purity of such microglia cultures is assessed on the basis of morphology and immunohistochemical analysis after isolation, and frequently shows some contamination with astrocytes. In addition, as whole rodent brain homogenates contain major blood vessels and frequently also the meninges, these cultures include CNS-associated macrophages or cells derived from the circulation that contaminate these preparations. In contrast, by sorting the cells, the definition and criteria for microglia isolation are determined beforehand. We sorted and cultured resident microglia based on CD11b expression. Although other cells also express CD11b like blood-derived monocytes/macrophages, a subset of dendritic cells, granulocytes and perivascular macrophages, contamination with these cells in our procedure is expected to be negligible for a number of reasons. First, dendritic cells and granuloctyes are hardly present in the brain parenchyma. If these cells would be present in the isolates, they would derive from the circulation. We demonstrated that the fraction of $CD11b^{-}CD45^{+}$ lymphocytes is very small, clearly indicating that the contribution of cells derived from the blood compartment is low. Accordingly, also the number of blood-derived monocytes/macrophages will be insignificant. Second, we and others have previously demonstrated that perivascular macrophages express high levels of MR.[163,183] However, even with the highly sensitive technique of qPCR, MR expression was not detectable in the microglia cultures, indicating that there was no significant contribution of these cells to the cultures. Finally, confirming previous observations,[184] microglia were smaller than choroid plexus macrophages, as seen in the forward versus side scatter profiles of the unpurified cells, as well as consistently seen in culture, corresponding with their morphology in the brain parenchyma. This finding indicates that with respect to the size of the cells, cultured microglia display a homogenous cell population, which is in line with data on mouse microglia.[184]

Besides microglia, choroid plexus cells were also sorted based on CD11b expression. Because a substantial amount of lymphocytes was detectable in the initial isolation, contamination of blood-derived macrophages or granulocytes can not be excluded. However, since the choroid plexus is highly enriched for macrophages, we expect that the majority of the cells are macrophages. Future experiments have to address the purity of this culture. Nevertheless, for the present study, choroid plexus cells were used as control for the microglia culture. In keeping with our previous study (Koning *et al*. submitted), we showed that CD200R and MR are induced

on choroid plexus macrophages following IL-4 stimulation. This suggests an IL-4 induced polarization of these cells and also confirms that the isolation and sorting procedure had no ill effect on IL-4 stimulation of macrophages.

Human microglia biology, especially in MS, is a largely undiscovered field. Because of their controversial association with many neurodegenerative diseases, it is very important to know whether activation of microglia can lead to polarization as seen in other types of macrophages. Under influence of IL-4, choroid plexus macrophages increased CD200R expression, but this was not demonstrated in microglia, although expression of the molecule was evident in these cells. MR is a commonly used marker for alternatively activated macrophages[172] and was indeed up-regulated in choroid plexus macrophages upon IL-4 stimulation, but again, not in microglia. Because MR mRNA levels were undetectable in microglia, MR expression might in addition be useful as a discriminator between macrophages and microglia in man. These data contradict a study on mouse microglia, demonstrating MR expression and up-regulation by IL-4 on these cells.[189,190] However, microglia were obtained 4 to 5 weeks following preparation of the neonatal brains, based on adherence. As stated above, it is possible that these cultures included macrophages derived from blood, perivascular and subarachnoidal spaces, meninges and choroid plexus and astrocytes that are claimed by this group to express MR as well.[189] From our data however, we will further address the usability of MR protein expression in cell sorting as a functional discriminator between human macrophages and microglia.

Although microglia are often referred to as 'macrophages of the brain', the preliminary data from the present study suggest that human microglia do not behave like macrophages in terms of classic or alternative macrophage polarization. On the other hand, HLA-DR expression levels indicated that the cultured microglia were in an activated state. This activation may have caused polarization in either direction. This is an interesting subject for further study. For example, would the activation state of microglia in culture change following soluble CD200 treatment, and would IL-4 treatment then induce polarization and enhance CD200R expression? Future studies will also have to confirm the presence of the receptor for IL-4 on these microglia, as well as its downstream pathway molecules STAT6 and PPARδ, that have been implicated in the induction of alternatively activated macrophages.[191,192]

CD200-CD200R interaction provides an essential inhibitory mechanism to maintain an immune suppressed environment in the brain.[111] Absence of CD200 in mice causes spontaneously activated microglia and results in accelerated onset of experimental autoimmune encephalomyelitis (EAE), the animal model for MS.[125] For such an important system it is peculiar that no regulators of CD200R, other than

IL-4 in monocyte-derived macrophages, have been identified. Also in our hands, none of the different pro- and anti-inflammatory compounds tested convincingly altered CD200 expression on neuroblastoma cells. A recent publication suggests that IL-4 enhanced neuronal CD200 expression.[193] However, the authors used a concentration of 20 µg/ml, 4000 times the concentration used in the present study, and overwhelmingly exceeding physiological concentrations.

In conclusion, the present data suggest that microglia resemble macrophages to a lesser extent than previously assumed. The expression of CD200R on human microglia and CD200 on neurons is confirmed but their regulation remains unidentified. Finally, this study demonstrates a functional technique of isolating, sorting and culturing microglia and provides a powerful tool to further study CD200R regulation in these cells as well as human primary microglia biology in general.

Balanced immunity by GITR-GITRL

6

GITR triggering induces expansion of both effector and regulatory CD4$^+$ T cells *in vivo*

Ronald W. van Olffen, Nathalie Koning, Klaas P.J.M. van Gisbergen, Felix M. Wensveen, Robert M. Hoek, Louis Boon, Jörg Hamann, René A.W. van Lier, Martijn A. Nolte

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Abstract

Glucocorticoid-induced tumor necrosis factor receptor family-related protein (GITR) is expressed on activated and regulatory T cells, but its role on these functionally opposing cell types is not fully understood. Here we describe that transgenic expression of GITR's unique ligand (GITRL) induces a prominent increase of both effector and regulatory $CD4^+$ T cells, but not $CD8^+$ T cells. Regulatory T cells from GITRL-transgenic mice are phenotypically activated and retain their suppressive capacity. The accumulation of effector and regulatory T cells is not due to enhanced differentiation of naïve T cells, but a direct result of increased proliferation. Functional consequences of increased numbers of both regulatory and effector T cells were tested in an autoimmune model and show that GITR stimulation is protective, as it significantly delays disease induction. These data indicate that GITR regulates the balance between regulatory and effector $CD4^+$ T cells by enhancing proliferation of both populations in parallel.

Introduction

Members of the TNF receptor (TNFR) superfamily are able to directly and indirectly affect the course of an immune response.[194] The glucocorticoid-induced tumor necrosis factor receptor family-related protein (GITR) is a member of this family and has been implicated in regulating both innate and adaptive immune responses.[195,196] Regulatory T cells are well-known for their expression of GITR, though this receptor is also expressed on activated non-regulatory T cells, as well as B cells, monocytes and macrophages, dendritic cells and mast cells.[196-200] Its ligand (GITRL) can be found on a variety of cells, including dendritic cells, macrophages and B cells.[200-202] GITRL is transiently up-regulated on these antigen presenting cells upon stimulation via the transcription factor NF-1[203] and it most likely exerts its main function during inflammatory responses. Indeed, agonistic antibodies and (cells expressing) recombinant GITRL enhance T cell proliferation *in vitro* upon TCR triggering,[198,204-207] which suggests that GITR acts as a costimulatory factor for T cells. GITR stimulation on regulatory T cells in coculture with effector T cells has been suggested to neutralize the suppressive capacity of regulatory T cells.[198,208,209] However, it was later shown that GITR ligation on regulatory T cells does not directly affect their suppressive capacity, but that GITR stimulation on non-regulatory T cells allows them to escape suppression by regulatory T cells.[210] GITR is not essential for regulatory T cell function, as regulatory T cells from $GITR^{-/-}$ mice display a normal capacity to suppress T cell proliferation *in vitro*.[211,212] This leaves unanswered what the function of GITR is on regulatory T cells.

Studies using $GITR^{-/-}$ mice showed that the absence of GITR was protective in several disease models, which was attributed to an impaired effector function of T cells.[213-215] Correspondingly, studies that have directly addressed the function of GITR on T cells *in vivo* by deliberate stimulation of the receptor with agonistic antibodies conclude that GITR has a pro-inflammatory role within the immune system through its costimulatory effects on T cells.[200,216-218] However, these antibodies have their limitations when studying the impact of GITR stimulation in complex disease models, in particular since anti-GITR antibodies have been reported to cause depletion of regulatory T cells.[200] Moreover, recent studies on the crystal structure of GITRL have revealed that this ligand can exist in multiple oligomerization states that depend on binding to the receptor,[219,220] and it therefore remains to be addressed whether crosslinking GITR with agonistic antibodies exerts the same downstream effects as signaling induced by membrane-bound GITRL. Thus, in order to properly address the consequence of direct GITR stimulation on T cell function *in vivo*, we generated transgenic (TG) mice in which GITRL is constitutively expressed on

B cells. Our findings demonstrate that GITR stimulation *in vivo* very effectively increases the absolute number of both effector and regulatory $CD4^+$ T cells through enhanced proliferation of both cell types. In agreement with increased regulatory T cell numbers, GITRL TG mice showed a marked delay in disease onset upon induction of experimental autoimmune encephalomyelitis (EAE), an experimental model for multiple sclerosis. We propose that GITR plays an important role in the regulation of both regulatory and effector $CD4^+$ T cell numbers *in vivo* by enhancing their turnover.

Methods

Generation of GITRL TG mice

cDNA encoding murine GITRL was obtained via PCR on total splenic cDNA and cloned into the pGEM-T plasmid (PROMEGA). This construct was digested with NotI and XhoI to obtain a 600 bp fragment containing the mGITRL cDNA, which was cloned into the NotI – XhoI site of a CD19-pC3 plasmid (kindly provided by Patrick Derksen, Academic Medical Center, The Netherlands), resulting in the GITRL expression construct under control of the human CD19 promoter. This construct was linearized via AatII digestion (see Fig. 1A) and microinjected into pronuclei of C57BL/6 fertilized oocytes and implanted into pseudopregnant female C57BL/6 mice. Transgenic (TG) founders were identified by PCR analysis of tail or ear DNA, using the following PCR primers: pC3s1 (5'-GCAGTGACTCTCTTAAGGTAGCC-3') and mGITRL4a (5'CTTGAGTGAAGTATAGATCAGTGTA-3'). Three GITRL TG founder lines (RW14, RW18, RW20) were propagated by mating with wild type C57BL/6 (WT) mice and offspring was tested for the presence of the transgene by PCR analysis of tail or ear DNA with the same primers.

Mice

GITRL TG mice were maintained on a C57BL/6 background and bred in the animal department of the Academic Medical Center (Amsterdam, The Netherlands) under specific-pathogen-free conditions. Mice were used at 6-24 weeks of age, age- and sex-matched within experiments and were handled in accordance with institutional and national guidelines. For measurement of *in vivo* T cell proliferation, mice were injected i.p. with 1 mg (+)-5-Bromo-2'deoxyuridine, (BrdU, Aldrich) and sacrificed for analysis 16 hours later.

Cell staining and flow cytometry

Single-cell suspensions were obtained by mincing the specified organs through 40 µm cell strainers (Becton Dickinson). Erythrocytes were lysed with an ammonium chloride solution and cells were subsequently counted using an automated cell counter (Casy, Schärfe System). Cells ($5x10^5$ - $5x10^6$) were collected in staining buffer (PBS with 0.5% bovine serum albumin (Sigma)) and stained for 30 minutes at 4˚C with antibodies in the presence of anti-CD16-CD32 (clone 2.4G2). The following fluorescently or biotin-labeled monoclonal antibodies (and clone names) were obtained from Pharmingen: anti-B220 (RA3-6B2), anti-CD3ε (145-2C11), anti-CD4 (L3T4), anti-CD8 (Ly-2), anti-CD62L (clone MEL-14), anti-CD69 (H1.2F3) or from eBioscience: anti-GITRL (ebioYGL386), anti-FoxP3 (NRRF-30), anti-GITR (DTA-1), anti-CD44 (IM7), anti-CTLA4 (4C10-4B9), anti-PD1 (RMP1-30), anti-CD134 (OX86), anti-CD27 (LG.7F9), anti-CD103 (M290), anti-CD45.1 (104 or A20). PE-conjugated anti-CD25 was obtained from Miltenyi Biotec. For the detection of biotinylated antibodies, streptavidin-PE (Caltag Laboratories, CA), streptavidin-APC (Pharmingen) or streptavidin-conjugated PerCP-Cy5.5 (Pharmingen) was used. Intracellular stainings for FoxP3 and/or BrdU were performed using FoxP3 Fixation/Permeabilization Concentrate and Diluent (eBioscience), according to the manufacturer's protocol. For BrdU staining, FITC-conjugated anti-BrdU/Dnase (Becton Dickinson) was added during the FoxP3 staining step and stained for 25 min at RT. Data were collected on a FACSCalibur or FACSCanto (Becton Dickinson) and were analyzed using FlowJo software (Treestar).

Intracellular cytokine staining

To determine direct *ex vivo* cytokine production, splenocytes were plated at $1x10^6$ cells/well in a 96-well round-bottom plate and stimulated with 1 ng/ml PMA and 1 µM ionomycin. After 2 hours incubation at 37°C, 1 µg/ml of the protein-secretion inhibitor Brefeldin A was added (Sigma) and cells were cultured for another 4 hours. Hereafter, cells were washed and stained for CD4 and CD8, followed by fixation and permeabilization (Becton Dickinson). Cells were then incubated for 30 min with fluorescently labelled antibodies against IFN-γ or IL-2 (eBioscience), thoroughly washed and analyzed by flow cytometry.

T cell proliferation assay

To analyze the effect of GITR engagement on the proliferative capacity of WT responder cells, T cells were enriched from spleens of WT mice by negative selection using $CD19^+$ beads (Miltenyi Biotec). T cell enriched splenocytes were labeled with 0.25 µM carboxyfluorescein succinimidyl ester (CFSE) in PBS at 37˚C for 10 minutes

and stimulated with 100 ng/ml anti-CD3 (clone 145-2C11) for 3 days in the presence of irradiated (10 Gray) WT or GITRL TG B cells with or without 200 U/ml IL-2. B cells were isolated by positive selection using $CD19^+$ beads (Miltenyi Biotec). For the analysis of the expression of CD25 and CD69, non-CFSE-labeled enriched WT T cells were used, stimulated similarly and analyzed after 1 day. To determine the effects of GITR ligation on IL-2 production *in vitro*, T cell enriched splenocytes were stimulated as described above, in the presence of 10 µg/ml blocking anti-CD25 antibody (clone PC61) to prevent IL-2 consumption. Culture supernatant was harvested after 1 day of stimulation and frozen at -20°C. The IL-2 ELISA (Becton Dickinson) was performed according to instructions from the manufacturer.

Regulatory T cell assay

Splenic $CD4^+CD25^-$ (responder cells) and $CD4^+CD25^{hi}$ (regulatory cells) T cells were isolated by cell sorting using a FACSAria (Becton Dickinson) and purity of sorted populations was consistently >96%. Responder cells were mixed with regulatory T cells at different ratios in 96-well tissue culture plates. The cells were stimulated with 10 µg/ml soluble anti-CD3 (clone 145-2C11) plus irradiated (10 Gray) WT splenocytes (APCs) at 37˚C for 72 hours. Hereafter, cells were pulsed for 16 hours with 1 µCi 3H-TdR ([Methyl-^{3}H]Thymidine, Amersham Pharmacia)/well, and incorporation of 3H-TdR was determined using a Beta Plate scintillation counter (Wallac, 1450 microbeta Plus Liquid Scintillation counter). Data are presented as percentage proliferation compared to maximum responder cell proliferation of triplicate assays.

MLPA

RNA isolation and MLPA analysis was performed as previously described with the murine apoptosis kit (RM002, MRC-HOLLAND) in collaboration with MRC-HOLLAND.[221]

Adoptive transfer of naïve T cells into WT and GITRL TG mice

For adoptive transfers, naïve ($CD25^-$) $CD4^+$ T cells were purified from spleens and peripheral lymph nodes of Ly5.1 mice by negative selection using the $CD4^+CD25^+$ regulatory T cell isolation kit (Miltenyi Biotec). Purified $CD4^+CD25^-$ cells (purity >90%) were labeled with 0.25 µM carboxyfluorescein succinimidyl ester (CFSE) in PBS at 37˚C for 10 min and injected after washing (± $1x10^6$ in 200 µl PBS) i.v. into WT and GITRL TG recipient mice. Distribution and phenotype of transferred cells was analyzed 3 days later by flow cytometry.

EAE induction

EAE was induced by s.c. immunization of mice in the hind flanks using 50 µg of MOG_{35-55} peptide in CFA containing 1 mg/ml heat-inactivated Mycobacterium tuberculosis (Difco) on day 0. Mice also received 200 ng of pertussis toxin (Sigma) i.v. on days 0 and 2. Disease severity was assessed according to the following scale: 0, no disease; 1, flaccid tail; 2, loss of hind leg spreading reflex; 3, hind limb weakness; 4, unilateral hind limb paralysis; 5, bilateral hind limb paralysis; 6, abdominal paralysis; 7, moribund; 8, dead. All mice were sacrificed 14 days following EAE induction after which brain and spinal cord was frozen in Tissue-Tek (Sakura Finetek, The Netherlands) at -80°C for immunohistochemical analysis.

Immunohistochemistry

Cryostat sections (8 µm) of spinal cord and brain of 4 WT and 4 TG mice were fixed in acetone, containing 1% H_2O_2 for 10 minutes. Then, sections were incubated with monoclonal rat anti-mouse antibodies to CD68 (a kind gift from Siamon Gordon, Oxford, UK, clone FE-11), CD4 and FoxP3 (eBioscience), diluted in PBS with 8% bovine serum albumine (BSA), 10% normal mouse serum (NMS) and 0.05% NaN_3 for 1

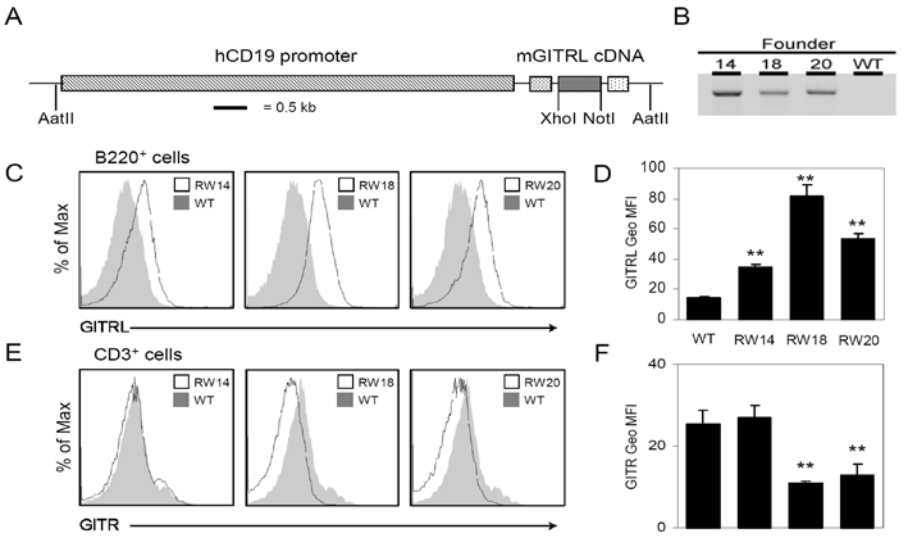

Fig. 1 Generation of B cell specific GITRL TG mice. (A) Schematic representation of the hCD19-mGITRL DNA construct. The human CD19 promoter (hatched box) is followed by a chimeric intron (white box), mGITRL cDNA (dotted box) and a poly A tail (black box). (B) PCR analysis of genomic tail DNA from WT or GITRL TG mice (founder lines RW14, 18 and 20). (C) Representative staining for GITRL on splenic $B220^+$ cells from WT, RW14, RW18 and RW20 mice and (D) expression as the average geometric mean fluorescence intensity (geoMFI) ± SD for 3 mice per group. (E) Representative staining for GITR expression on splenic $CD3^+$ cells from WT, RW14, RW18 and RW20 mice and (F) expression as the average geoMFI ± SD. Asterisks denote significant differences (** $p<0.005$).

hour at 4°C. After washing in PBS, the sections were incubated with anti-rat HRP diluted in PBS/8% BSA/10% NMS/0.05% NaN_3/350 mM NaCl. Staining was visualized with DAB (Sigma Laboratories, St. Louis, MO, USA) applied for 10 minutes. Sections were counterstained with haematoxylin for 30 seconds, dehydrated and mounted in entallan (Merck, Darmstadt, Germany). As negative controls, primary antibodies were either omitted or substituted with an isotype control antibody. No immunoreactivity was seen for all negative controls.

Statistical analysis

Statistical analysis of the data was performed using the unpaired Student's t-test or Wilcoxon rank-sum test where mentioned. For the EAE experiments, effect on mean clinical score was assessed by calculating the area under the curve using the trapezoidal rule, followed by the Wilcoxon rank-sum test. Differences in cumulative incidence were analyzed on a per day basis, using a χ^2 test of a contingency table.

Results

Generation of B cell specific GITRL TG mice

To study the function of GITR on T cells *in vivo*, we generated B cell specific GITRL TG mice by expressing GITRL cDNA under control of the human CD19 promoter (Fig. 1A). Through microinjection of fertilized oocytes, we acquired three founder lines (RW14, RW18 and RW20), which were identified by genomic PCR analysis (Fig. 1B). GITRL TG mice were fertile, born at expected Mendelian frequencies and appeared as healthy as their littermate controls. Flow cytometry showed that GITRL was indeed significantly expressed on B cells in all founder lines, with highest expression on the RW18 line (Fig. 1C-D). As receptor downregulation upon ligand stimulation is a hallmark for TNFR-superfamily members, we determined the expression of GITR on T cells. T cells from GITRL TG mice showed decreased GITR expression compared to WT mice, which correlated with the level of GITRL expression (Fig. 1E-F). This indicates that GITR is indeed functionally engaged by its ligand in these mice. The data shown below are obtained from experiments with the GITRL TG RW18 line, though a similar, but less pronounced phenotype was also found in the other founder lines (data not shown).

GITRL TG mice have more $CD4^+$ effector memory-like and regulatory type T cells

To establish the effects of GITR triggering *in vivo*, we analyzed the primary and secondary lymphoid organs of GITRL TG mice. T cell differentiation in the thymus

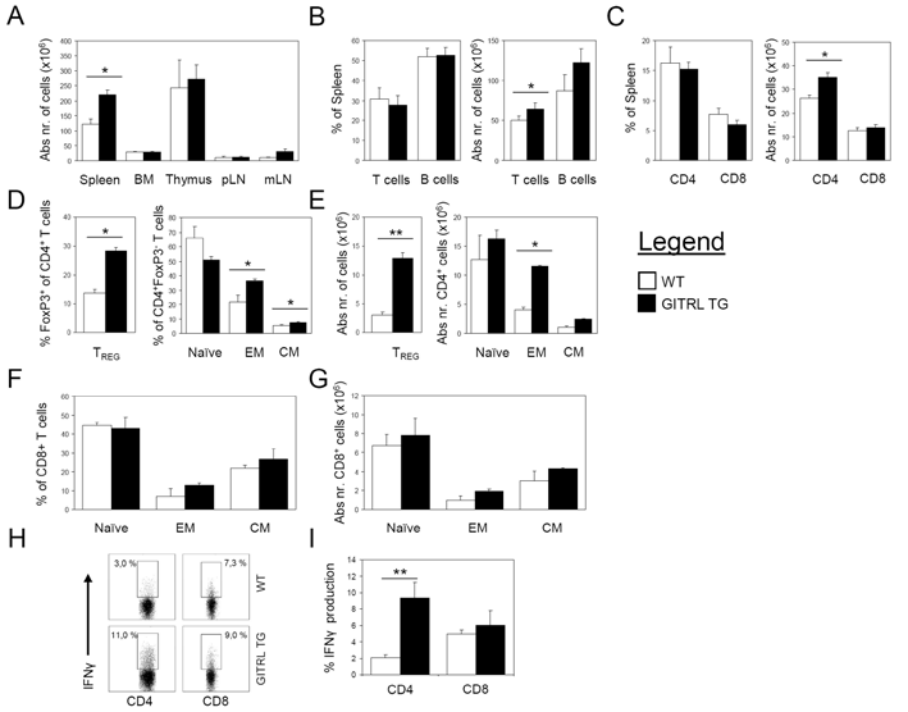

Fig. 2 GITRL TG mice have more effector and regulatory type CD4$^+$ T cells. (A) Absolute number of cells in spleen, bone marrow (BM), thymus, peripheral (pLN) and mesenteric (mLN) lymph nodes in 4-8 weeks old WT (white bar) and GITRL TG (black bar) mice. Percentage and absolute number of (B) T and B cells or (C) CD4$^+$ and CD8$^+$ T cells in the spleen of WT and GITRL TG mice. (D, E) Percentage and absolute number of splenic regulatory (FoxP3$^+$) and non-regulatory (FoxP3$^-$) CD4$^+$ T cells in WT and GITRL TG mice. Non-regulatory CD4$^+$ T cells were subdivided in naïve (CD44$^-$CD62L$^+$), effector memory (EM) (CD44$^+$CD62L$^-$) and central memory (CM) (CD44$^+$CD62L$^+$) cells. (F, G) Percentage and absolute number of naïve, EM and CM cells of splenic CD8$^+$ T cells. Production of IL-2 (H) and IFN-γ (I) by CD4$^+$ or CD8$^+$ T cells in WT and GITRL TG mice after stimulation with PMA/ionomycin. Asterisks denote significant differences (* $p<0.05$; ** $p<0,005$). Data represent the average value ± SD of 3-5 mice and are representative for 2-4 independent experiments.

of these mice was comparable to WT littermates (data not shown), as was cellularity of bone marrow, thymus, peripheral and mesenteric lymph nodes (Fig. 2A). However, spleens of GITRL TG mice contained significantly more leukocytes than WT mice (Fig. 2A). This increase was due to elevated numbers of CD4$^+$ T cells (Fig. 2B-C), whereas numbers of B cells and CD8$^+$ T cells were not significantly altered (Fig. 2B-C). Analysis of FoxP3 expression indicated that a substantial part of this increase in CD4$^+$ T cells could be attributed to an enlarged regulatory T cell compartment, as up to three times more CD4$^+$FoxP3$^+$ regulatory T cells were present in the spleens of GITRL TG mice (Fig. 2D-E). Phenotypic analysis of FoxP3$^-$CD4$^+$ T cells indicated that the non-regulatory fraction was also affected in GITRL TG mice, as

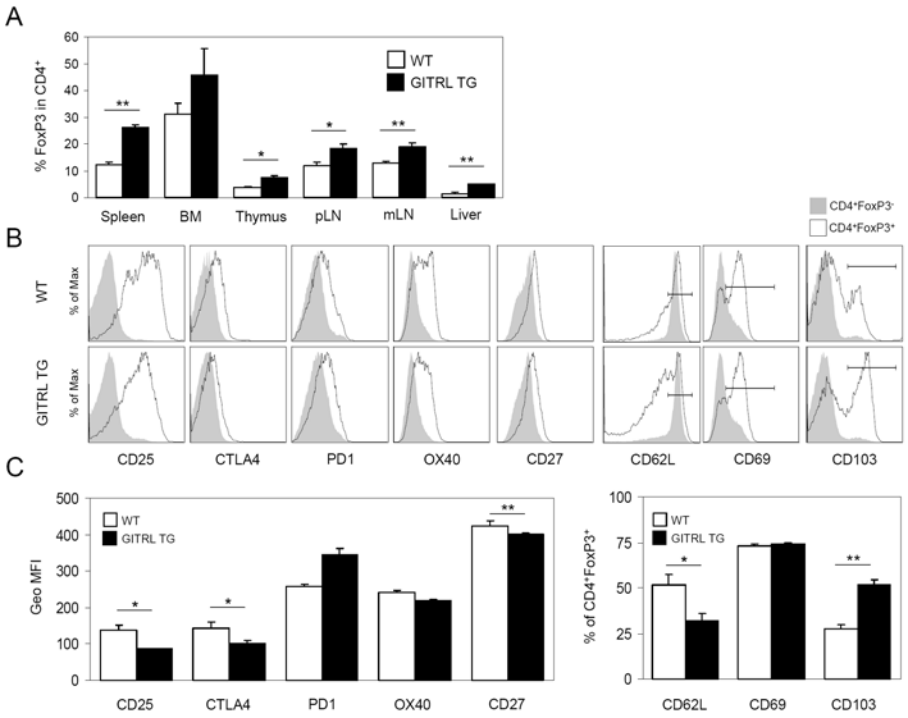

Fig. 3 Systemic increase of regulatory T cells via GITR signaling. (A) The percentage of FoxP3$^+$ cells within the CD4$^+$ T cell compartment in spleen, bone marrow (BM), thymus, peripheral (pLN) and mesenteric (mLN) lymph nodes and liver in WT and GITRL TG mice. (B) Phenotypic analysis of splenic FoxP3$^-$ (filled graph) and FoxP3$^+$ (open graph) CD4$^+$ T cells for surface expression of CD25, CTLA4, PD1, OX40, CD27, CD62L, CD69 and CD103 in WT and GITRL TG mice. (C) Average intensity (geoMFI ± SD) or percentage positive cells (% ± SD) of these molecules on FoxP3$^+$CD4$^+$ T cells. Asterisks denote significant differences (* $p<0.05$, ** $p<0.005$). Data are representative for 2 independent experiments with each at least 3 mice per group.

significantly more CD4$^+$ T cells with an effector memory-like (CD44$^+$CD62L$^-$) and central memory-like (CD44$^+$CD62L$^+$) phenotype were identified (Fig. 2D-E). This increase of CD4$^+$ T cells with either a regulatory or a memory-like phenotype in GITRL TG mice apparently did not develop at the cost of the naïve CD4$^+$ population, since absolute numbers of naïve CD4$^+$ T cells were comparable with WT littermates (Fig. 2E). No differences were found for CD8$^+$ T cells with respect to their naïve, effector memory-like and central memory phenotype (Fig. 2F-G). Corroborating the specific increase in CD4$^+$ effector T cells in GITRL TG mice, splenocyte stimulation with PMA-ionomycin showed increased production of the effector cytokines IL-2 (Fig. 2H) and IFNγ (Fig. 2 I-J) by CD4$^+$, but not CD8$^+$ T cells. These data thus indicate that GITR triggering *in vivo* enhanced the number of both regulatory and effector CD4$^+$ T cells.

Distribution and activation status of regulatory T cells in GITRL TG mice

To determine whether the strong increase of regulatory T cells in GITRL TG mice was restricted to the spleen, we analyzed the presence of these cells in bone marrow, thymus, peripheral and mesenteric lymph nodes and liver in these mice. We found that GITRL TG mice have a systemic increase in regulatory T cell numbers, as all analyzed compartments, except for the bone marrow, showed a significantly higher fraction of $FoxP3^+$ $CD4^+$ cells compared to WT mice (Fig 3A).

Next, we analyzed the activation status of splenic regulatory and non-regulatory T cells. Apart from the described changes in CD44 and CD62L expression (see Fig. 2D-E), non-regulatory $CD4^+$ T cells in GITRL TG mice were comparable with their WT counterparts on the basis of several other costimulatory and activation molecules (Fig 3B). On the other hand, we found that $CD4^+FoxP3^+$ regulatory T cells from GITRL TG mice were quite distinct from their counterparts in WT mice, as they expressed lower levels of CD25, CD62L and CTLA-4 (Fig 3B-C). In addition, the expression of PD-1 was increased in GITRL TG mice, while a large fraction of regulatory T cells from GITRL TG mice expressed the adhesion molecule CD103 (αE integrin) on their surface (Fig 3B-C). The expression of OX40, CD27 and CD69 was comparable to WT mice (Fig 3B-C). Based on these characteristics, in particular the decreased levels of CD62L and the high percentage of $CD103^+$ cells, we conclude that constitutive GITR stimulation not only leads to more regulatory T cells, but also stimulates their differentiation towards an effector phenotype.[222]

GITR engagement in vivo does not affect the suppressive capacity of regulatory T cells

To determine if the altered phenotype of regulatory T cells in GITRL TG mice mirrored a change in their function, we performed *in vitro* proliferation assays, in which WT responder T cells ($CD4^+CD25^-$) were stimulated with anti-CD3/CD28 in the presence of increasing numbers of regulatory T cells ($CD4^+CD25^+$) from WT or GITRL TG mice. From these experiments it can be concluded that regulatory T cells from GITRL TG are fully capable to suppress responder T cell proliferation and were equally anergic as regulatory T cells from WT mice (Fig. 4A). This conclusion challenges previous reports, which have suggested that GITR stimulation on regulatory T cells is sufficient to abrogate their suppressive capacity.[198,223]

We also analyzed the susceptibility of GITRL TG vs WT derived responder T cells to the suppressive capacity of WT regulatory T cells, as it has been reported that GITR stimulation allows T cells to escape suppression by regulatory T cells.[210] These experiments revealed that responder T cells from GITRL TG mice could still be adequately suppressed by regulatory T cells (Fig. 4B), thereby indicating that chronic GITR stimulation *in vivo* is not sufficient to induce an enduring state of insensitivity

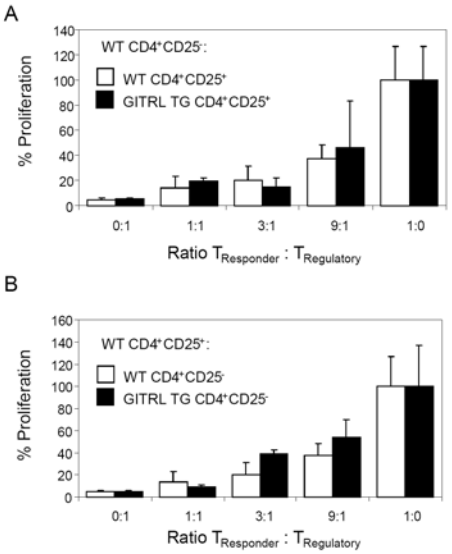

Fig. 4 Regulatory T cell function of WT and GITRL TG mice. (A) Ability of purified WT and GITRL TG regulatory ($CD4^+CD25^+$) T cells to suppress WT responder ($CD4^+CD25^-$) T cells. (B) Ability of WT regulatory T cells to suppress proliferation of WT and GITRL TG responder T cells. Cells were cultured at different ratios for 4 days with 10 µg/ml soluble anti-CD3 mAb in the presence of irradiated WT splenocytes as antigen presenting cells; for the final 16 hours [^{3}H]thymidine was added and incorporation was measured. Data are depicted as the percentage proliferation compared to responder T cells alone (average of triplicate wells ± SD) and are representative of 2 independent experiments.

to regulatory T cell activity. Instead, it rather suggests that this previously described capacity of GITR is only effective when given together with TCR stimulation.[210]

GITR engagement does not enhance the survival profile of T cells

To investigate whether the increase in effector memory-like and regulatory type T cells could be explained by an increased survival potential mediated through GITR signaling, we examined the expression profile of approximately 40 pro- and anti-apoptotic proteins using an advanced PCR approach called multiplex ligation-dependent amplification (MLPA).[221] Naïve ($CD25^-CD62L^+CD44^-$), effector ($CD25^-CD62L^-CD44^+$) and regulatory ($CD25^{hi}$) type $CD4^+$ T cells from WT and GITRL TG mice were purified by cell sorting and their mRNA was isolated and subjected to MLPA analysis. These data revealed no obvious differences in the apoptotic gene expression profile of naïve, effector and regulatory type T cells for WT and GITRL TG mice (Supplemental data, Table 1). The only exception was the expression of granzyme B, which was significantly increased in effector $CD4^+$ T cells of GITRL TG compared to WT mice. This finding is indicative for enhanced effector T cell formation in GITRL

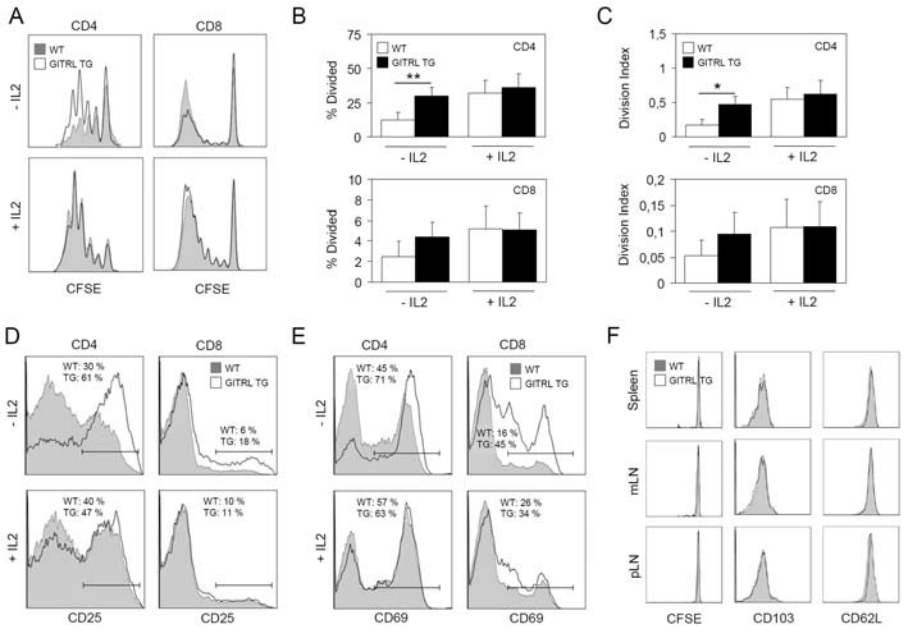

Fig. 5 GITR costimulation *in vitro* enhances CD4$^+$ T cell proliferation and IL-2 production. (A) Anti-CD3-induced proliferation of CFSE-labelled WT T cells cultured for 3 days in the presence of WT (filled graph) or GITRL TG (open graph) B cells with or without IL-2. Triplicate wells analyzed for (B) the average precursor frequency and (C) the average division index (i.e. the number of divisions that the dividing population underwent). (D) IL-2 concentration in supernatants (average of triplicate wells ± SD) after stimulating WT T cells as in (A) for 24 hours in the presence of a blocking antibody against CD25 to prevent IL-2 consumption. Expression of CD25 (E) and CD69 (F) on CD4$^+$ T cells stimulated as in (A) after 24 hours. (G) Naïve WT CD4$^+$CD25$^-$ T cells from Ly5.1 mice were CFSE-labeled and injected intravenously in WT (filled graph) or GITRL TG (open graph) mice. Donor cells, gated on CD45.1$^+$CD4$^+$ T cells, were analyzed 3 days after transfer for expression of CFSE, CD103 and CD62L expression. A representative staining from 3 mice per group is shown. Asterisks denote significant differences (* $p<0.05$, ** $p<0.005$).

TG mice and corresponds to the increased number of IFN-γ-producing CD4$^+$ T cells in these mice (Fig. 2I). Based on these data it is highly unlikely that basic changes in cell survival underlie the increase of effector and regulatory T cells in GITRL TG mice.

GITR costimulation enhances CD4$^+$ T cell proliferation and IL-2 production

Next, we set out to determine the effects of GITR triggering on T cell proliferation. CFSE-labeled T cells from WT mice were stimulated with suboptimal concentrations anti-CD3 in a 1:1 ratio with WT or GITRL TG irradiated B cells for a period of 3 days. We found that increased GITRL availability enhanced CD4$^+$ T cell proliferation, but did not affect CD8$^+$ T cell proliferation (Fig. 5A). The enhanced proliferation of CD4$^+$

T cells via GITR engagement was no longer apparent when extra IL-2 was added to these cultures, indicating that GITR engagement affects the early proliferative capacity of $CD4^+$ T cells. We found that increased GITR ligation raised the percentage of $CD4^+$ T cells entering cell division (Fig. 5B), as well as the number of divisions that these cells underwent (Fig. 5C). Since the addition of IL-2 enhanced T cell proliferation to a similar extent as the addition of GITRL TG B cells, we questioned whether GITR stimulation induced IL-2 production. Indeed, when WT T cells were stimulated with anti-CD3, the addition of GITRL TG B cells induced almost 3-fold more IL-2 than WT B cells (Fig. 5D). Moreover, GITR ligation increased the expression of CD25 and CD69 on $CD4^+$ T cells, confirming the enhanced IL-2 production and increased activation induced by GITR stimulation (Fig. 5E-F).

To determine if constitutive GITR triggering alone was sufficient to induce activation and/or proliferation of naïve T cells *in vivo*, we isolated naïve non-regulatory $CD4^+CD25^-$ T cells from $Ly5.1^+$ WT donor mice, labeled them with CFSE and transferred them into WT or GITRL TG ($Ly5.2^+$) recipients. Three days after transfer, naïve T cells transferred to both WT and GITRL TG mice showed no CFSE dilution and did not alter their expression levels of CD62L or CD103 (Fig. 5G). Thus, despite the fact that GITRL TG mice contained more T cells with an effector memory-like phenotype, these data imply that stimulation through GITR alone is not sufficient to induce activation or proliferation of naïve T cells. Yet, when TCR-triggering is provided, GITR stimulation enhanced the production of IL-2 and increased proliferation of $CD4^+$ T cells *in vitro*.

GITR engagement in vivo increases the proliferation of effector and regulatory $CD4^+$ T cells

As GITR engagement could directly and specifically enhance $CD4^+$ T cell proliferation, we investigated the proliferative capacity of $CD4^+$ T cells in WT and GITRL TG mice *in vivo*. As measured by Ki-67 expression, GITRL TG mice had more non-regulatory T cells ($CD4^+FoxP3^-$) in cell cycle in the spleen than WT mice (24% ± 2.2 in GITRL TG mice vs 14% ± 1.2 in WT mice) (Fig 6A-B). The fraction of regulatory T cells ($CD4^+FoxP3^+$) that stained positive for Ki-67 was not significantly different between WT and GITRL TG mice, but the fraction of Ki-67^+ cells within the regulatory T cell compartment is already high (~30%) in WT mice (Fig. 6A-B).

To directly assess T cell proliferation *in vivo*, WT and GITRL TG mice were injected i.p. with BrdU and incorporation of this compound was analyzed 16 hours later (Fig. 6C-D). Within the non-regulatory $CD4^+$ T cell compartment, we found that GITRL TG mice contained more $BrdU^+$ cells than WT mice and this increase was most pronounced in the $CD62L^-$ effector fraction (Fig. 6C-E). A GITR-mediated increase in

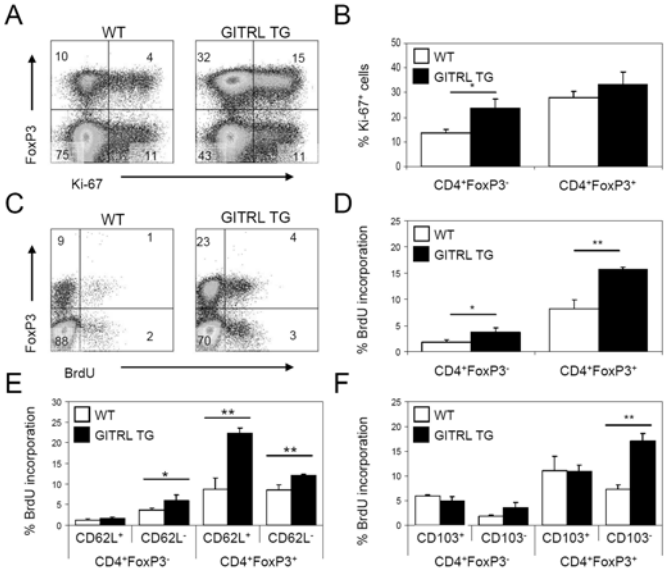

Fig. 6 GITR triggering enhances proliferation of effector and regulatory T cells *in vivo*. (A) Representative intracellular staining for FoxP3 and Ki-67 on splenic $CD4^+$ T cells from WT and GITRL TG mice and (B) the percentage Ki-67^+ cells of $FoxP3^-$ and $FoxP3^+$ $CD4^+$ T cells (average ± SD). (C) Representative intracellular staining for BrdU and FoxP3 on splenic $CD4^+$ T cells from WT and GITRL TG mice, 16 hours after i.p. injection of 1 mg BrdU. (D) The percentage $BrdU^+$ cells of $FoxP3^-$ and $FoxP3^+$ $CD4^+$ T cells (average ± SD). Characterization of proliferating $FoxP3^-$ and $FoxP3^+$ $CD4^+$ T cells based on CD62L (E) or CD103 (F) expression. The percentage of $BrdU^+$ cells in each fraction is depicted for WT (white bar) and GITRL TG (black bar) mice (average ± SD). Data are representative for 2 independent experiments with each at least 3 mice per group. Asterisks denote significant differences (* $p<0.05$, ** $p<0.005$).

proliferation was even more profound for regulatory T cells, as the percentage of $CD4^+FoxP3^+$ T cells that had incorporated BrdU had more than doubled compared with WT littermates (Fig 6C-D). In this case, it was the $CD62L^+CD103^-$ fraction of regulatory T cells that showed the most profound increase in BrdU incorporation (Fig. 6E-F). No effect of GITRL overexpression on BrdU incorporation was found in $CD8^+$ T cells (data not shown). Overall, these data indicate that GITR affects the numbers of regulatory T cells as well as the memory/effector pool of non-regulatory $CD4^+$ T cells *in vivo* by regulating their proliferation.

Enhanced GITR ligation delays experimental autoimmune encephalomyelitis

To examine the significance of the expansion of both regulatory and effector $CD4^+$ T cells on a complex immune response *in vivo*, GITRL TG mice were subjected to an experimental model for multiple sclerosis, experimental autoimmune encephalomyelitis (EAE). We selected this model as it induces autoimmunity by selective

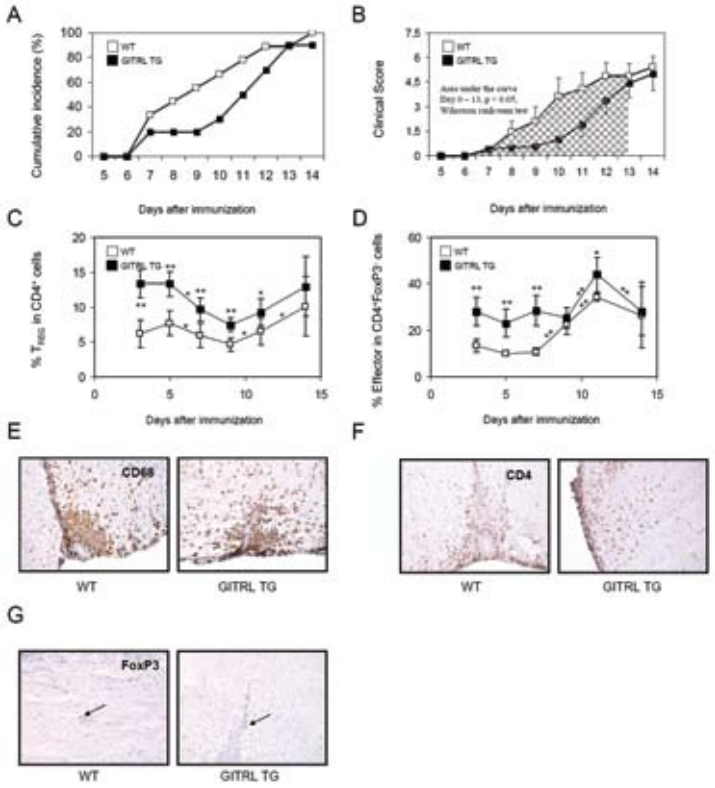

Fig. 7 Delayed EAE induction through GITR ligation. (A) The cumulative incidence and (B) the average clinical score following EAE induction in WT and GITRL TG mice. Experiment depicted contains 10 animals per group and is representative of 2 independent experiments. Significant differences ($p<0.05$) of the area under the curve were determined by Wilcoxon rank-sum test (highlighted area). (C) Percentage regulatory ($FoxP3^+$) and (D) non-regulatory effector ($FoxP3^-CD62L^-$) T cells within the $CD4^+$ compartment was determined in peripheral blood following immunization (day 0) and pertussis toxin injection (day 0 and 2) of WT and GITRL TG mice. Data represent the average ± SD of 4 mice. Asterisks denote significant differences between WT and GITRL TG mice on a particular day (* $p<0.05$, ** $p<0.005$). Black dots denote significant differences between consecutive days for either group (• $p<0.05$, •• <0.005). (E) Cellular infiltrates in brain and spinal cord were analyzed for mice with a clinical score of five and in which disease onset occurred four to five days prior to experimental endpoint. Representative immunohistochemical staining of the spinal cord of WT and GITRL TG mice shows the presence of (F) $CD68^+$ macrophages (G) $CD4^+$ T cells and (H) $FoxP3^+$ regulatory T cells on a haematoxylin background staining (20x magnification). Arrow indicates a single $FoxP3^+$ cell.

depletion and loss of function of regulatory T cells through treatment of immunized mice with pertussis toxin, which probably facilitates autoreactive T cells to develop and cause nerve damage in the central nervous system (CNS).[224,225] It is therefore conceivable that GITRL TG mice would be protected from disease (i.e. limb paralysis), because they have more regulatory T cells, but it could also be that

they would display enhanced susceptibility to EAE than WT mice because of their increased effector T cell formation. We found that regulatory mechanisms dominated the EAE response in GITRL TG mice, as they had a significant delay in disease onset compared to WT mice, which correlated with a lower clinical score (Fig. 7A-B). Although disease was delayed in GITRL TG mice, it was not inhibited, as the cumulative incidence and clinical score were similar between both groups at the experimental endpoint (Fig. 7A-B), suggesting that autoreactive cells did develop in these mice.

To gain insight in the relative contribution of regulatory and effector $CD4^+$ T cells in disease development, we examined the levels of both cell types in circulation following EAE induction. During the first 9 days, the fraction of regulatory $CD4^+$ T cells decreased in both groups of mice, but GITRL TG mice continuously displayed more regulatory T cells in circulation than WT mice (Fig. 7C). Both GITRL TG and WT mice showed a disease-related increase of effector type $CD4^+$ T cells in peripheral blood, but this occurred later in GITRL TG than in WT mice, which correlates with the observed delay in disease development (Fig. 7D). Finally, to investigate whether the observed differences in EAE signified merely a difference in time or also in quality of the immune response, we analyzed the cellular infiltrates in the central nervous system of mice with a similar clinical score. GITRL TG and WT mice showed a comparable influx of macrophages (Fig. 7E) and $CD4^+$ T cells (Fig. 7F) in the central nervous system and the influx of $FoxP3^+$ cells was equally low in both groups (Fig. 7G). Therefore, these data suggest that GITR engagement does not change the quality of this autoimmune response, but rather delays disease development by delaying the generation of disease-related effector type $CD4^+$ T cells.

Discussion

Since its discovery in 1997, GITR has been the focus of many studies that address its biological function in cellular immunology.[198,226-229] These studies have indicated that GITR has costimulatory effects during T cell activation, but it is still not fully understood at what level GITR triggering affects both T cell activation and regulatory T cell function and how this influences immune responses *in vivo*. Here we describe that *in vivo* GITR stimulation through its natural ligand increased absolute numbers of both effector and regulatory type $CD4^+$ T cells, which was not at the expense of the naïve $CD4^+$ T cell pool. Detailed analysis revealed that this increase was a direct consequence of enhanced proliferation of both effector and regulatory type $CD4^+$ T cells in GITRL TG mice. GITR ligation *in vivo* did not affect the anergic state of

regulatory T cells *in vitro*, nor did it influence the suppressive function of regulatory T cells. These data are congruent with the phenotype of GITR$^{-/-}$ mice, which have normally functioning regulatory T cells, but slightly fewer absolute numbers.[230] *In vitro* studies have shown that agonistic anti-GITR antibodies can induce proliferation of regulatory T cells in an IL-2 dependent manner, also without affecting their suppressive activity.[198,210] We found that GITRL expression on B cells increased IL-2 production by CD4$^+$ T cells *in vivo* (Fig. 2H) and *in vitro* (Fig. 5A), which is most likely a direct effect, as GITR crosslinking with antibodies can induce IL-2 production through TRAF-5 mediated NF-κB activation.[231] These results have two important implications for our understanding of the biological function of GITR on T cells. First, since regulatory T cells depend on exogenous IL-2 for their proliferation,[232] these findings indicate that GITR drives proliferation of both regulatory and effector T cells through the induction of IL-2 from the latter. Second, it explains why GITR stimulation on non-regulatory T cells allows them to escape suppression by regulatory T cells,[210] since it was recently shown that regulatory CD4$^+$ T cells exert their suppressive function through consumption of IL-2 produced by activated T cells, leading to apoptosis of the latter.[233] Since GITR triggering increases the production of IL-2, it allows them to escape or delay this cytokine deprivation-induced apoptosis. These implications fit in a previously postulated model for GITR function,[200] in which it was also suggested that when GITRL expression decreases at the end of an immune response, this would render effector T cells susceptible to suppression by an expanded, activated regulatory T cell pool. Transgenic GITRL expression does not allow us to test this hypothesis in our system, but it is worth following up this idea, as it implies that GITR is indirectly involved in termination of a T cell response.

Detailed analysis revealed that GITR ligation *in vivo* modified the expression of several key proteins expressed by regulatory T cells (Fig. 3). We found that the IL-2 receptor is down-regulated on regulatory T cells of GITRL TG mice, which is most likely a direct consequence of increased IL-2 consumption driving enhanced proliferation.[232] This is in agreement with recent findings that homeostatically proliferating regulatory CD4$^+$ T cells *in vivo* express lower levels of the IL-2 receptor than non-proliferating cells.[234] Furthermore, GITRL TG mice contained more regulatory T cells with an activated phenotype, expressing low levels of CD62L and high levels of CD103 (Fig. 3).[222] This is interesting, because we found that BrdU predominantly incorporated in the CD62L$^+$ and CD103$^-$ population of regulatory T cells in GITRL TG mice (Fig. 6). This would thus indicate that GITR ligation induces proliferation of CD62L$^+$CD103$^-$ regulatory T cells and that during this proliferation they become activated and accumulate as CD62L$^-$CD103$^+$ regulatory T cells. This would be in agree-

ment with an earlier study, which described that regulatory T cells with a high turnover downmodulate CD62L after several cell divisions.[235] Since CD62L is required for high endothelial venule dependent lymphocyte entry into lymph nodes and CD103 is an integrin necessary for the homing and retention of cells at inflammatory sites, these data suggest that regulatory T cells in GITRL TG mice are more prone to enter (inflamed) peripheral tissues than secondary lymphoid organs compared to their WT counterparts. Indeed, we found that liver and bone marrow of GITRL TG mice accumulate more $CD62L^{-}CD103^{+}$ regulatory T cells than WT mice (data not shown), but since the supply of regulatory T cells is also increased in these mice, it requires more specific migration experiments to adequately address this issue.

An intriguing finding from our analysis of GITRL TG mice is that the functional consequences of GITR engagement were restricted to $CD4^{+}$ T cells, as no effects on the proliferation or effector cell formation of $CD8^{+}$ T cells could be detected, neither *in vitro* nor *in vivo* (Fig. 2, 5A-C and data not shown). This is in contrast with other studies in which a role for GITR on $CD8^{+}$ T cell responses was demonstrated, using agonistic GITR antibodies or $GITR^{-/-}$ mice.[236-238] We found that both $CD4^{+}$ and $CD8^{+}$ T cells in GITRL TG mice had down-modulated surface expression of GITR compared to WT mice (Fig. 1 and data not shown), which indicates that GITR was functionally engaged by its ligand on both cell types. In WT mice, GITR expression is higher on $CD4^{+}$ non-regulatory T cells than on $CD8^{+}$ T cells[239] (and data not shown), which could be the reason why GITRL expression has a stronger effect on $CD4^{+}$ T cells than $CD8^{+}$ T cells. This might also relate to the finding that the costimulatory effect of GITR crosslinking with an anti-GITR antibody is apparent at a lower anti-CD3 concentration in $CD4^{+}$ T cells than in $CD8^{+}$ T cells.[210] Moreover, GITR upregulation following T cell activation is dependent on CD28 engagement in $CD4^{+}$, but not $CD8^{+}$ T cells.[210,238,240] Thus, although GITR functions on both $CD4^{+}$ and $CD8^{+}$ T cells, it is differently regulated in these subsets. In our hands, deliberate triggering of GITR on $CD8^{+}$ T cells *in vivo* by its natural ligand clearly does not translate into functional consequences, or at least not as strong as the effects found on $CD4^{+}$ T cells.

The synchronized expansion of regulatory and effector $CD4^{+}$ T cells that is induced upon GITR stimulation might seem contradictory for protective immunity, as these cell types obviously have opposite functions. However, recent *in vivo* studies have shown that regulatory T cells expand with similar kinetics as effector $CD4^{+}$ T cells upon HSV-2 infection[241] or immunization with Freund's complete adjuvants,[242] so that their ratio remains relatively constant. Coincident expansion of regulatory and effector T cells could be a direct consequence of responsiveness of regulatory T cells to IL-2 produced by effector T cells[243] and our data suggest that GITR could play a role in this process. The clinical consequence of an immune response might

even depend on this ratio, as the experimental induction of both adjuvant arthritis and type 1 diabetes correlates with an increased ratio of effector versus regulatory T cells.[244-246] The same might apply for the EAE model, as depletion of regulatory T cells resulted in enhanced disease progression and severity.[247] We found that the delay in disease induction observed in GITRL TG mice correlated with a delay in the increase of effector $CD4^+$ T cells in circulation (Fig. 7C-D). As no differences were observed in final disease severity nor cellular infiltrates in the brain parenchyma, these observations suggest that GITR stimulation sets a threshold for T cell activation and can delay autoimmunity by regulating the numbers of both regulatory and effector T cells.

In conclusion, we have shown that GITR serves as a costimulatory molecule in that it induces proliferation of regulatory as well as effector $CD4^+$ T cells *in vivo*. We suggest that upregulation of GITRL on antigen presenting cells during the initiation of an immune response, through the increase of pro-inflammatory stimuli, enhances IL-2 production and thereby the proliferation of cognate $CD4^+$ T cells, which also makes them less susceptible to suppression by regulatory T cells. At the same time, GITRL expression during this early phase induces the expansion of regulatory T cells, aided by the presence of exogenous IL-2 from proliferating non-regulatory T cells. These regulatory T cells might be important to re-establish the status quo of the immune system at later stages of the response.

Acknowledgments

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Supplemental data

Table 1. MLPA analysis on CD4+ T cells from WT and GITRL TG mice

Family		MLPA probe	Naïve T cells			Effector T cells			Regulatory T cells		
			WT mice	GITRL TG mice	Differential expression (Log2)	WT mice	GITRL TG mice	Differential expression (Log2)	WT mice	GITRL TG mice	Differential expression (Log2)
Anti-apoptotic		Bcl-W	0,00 ± 0,00	0,05 ± 0,05	0	0,08 ± 0,07	0,19 ± 0,03	1,272	0,10 ± 0,01	0,22 ± 0,07	1,136
		Bcl-Xl	1,34 ± 0,03	1,13 ± 0,05	-0,246	2,82 ± 0,21	2,07 ± 0,17	-0,446	3,21 ± 0,13	2,50 ± 0,29	-0,361
		Bcl-2	5,10 ± 0,40	5,81 ± 0,43	0,187	2,68 ± 0,30	2,34 ± 0,20	-0,195	7,05 ± 0,66	3,81 ± 0,95	-0,888
		A1a, A1b & A1d	4,44 ± 0,32	5,06 ± 0,53	0,189	15,04 ± 0,80	15,80 ± 1,22	0,071	9,34 ± 1,43	14,45 ± 0,72	0,629
		Mcl-1	5,13 ± 0,34	5,64 ± 0,26	0,137	4,03 ± 0,70	4,13 ± 0,24	0,034	5,44 ± 0,57	4,48 ± 0,74	-0,281
Pro-apoptotic	BH3-only	Bid	1,46 ± 0,05	1,48 ± 0,06	0,022	1,75 ± 0,32	2,01 ± 0,13	0,200	1,77 ± 0,20	1,77 ± 0,21	-0,007
		Bik	0,16 ± 0,02	0,12 ± 0,01	-0,369	0,79 ± 0,34	0,25 ± 0,01	-1,647	0,10 ± 0,01	0,08 ± 0,08	-0,310
		Bim	2,60 ± 0,21	2,49 ± 0,16	-0,063	1,78 ± 0,59	1,38 ± 0,11	-0,372	2,27 ± 0,42	1,03 ± 0,23	-1,137
		BMF	0,16 ± 0,03	0,11 ± 0,10	-0,534	0,27 ± 0,10	0,10 ± 0,10	-1,424	0,23 ± 0,12	0,05 ± 0,08	-2,274
		PUMA	0,86 ± 0,13	0,83 ± 0,05	-0,045	0,53 ± 0,07	0,66 ± 0,04	0,322	1,04 ± 0,20	1,03 ± 0,14	-0,006
		Noxa	0,00 ± 0,00	0,23 ± 0,05	0	0,44 ± 0,11	1,12 ± 0,04	1,337	0,35 ± 0,05	1,03 ± 0,20	1,572
		Bad	0,94 ± 0,08	0,83 ± 0,08	-0,180	0,99 ± 0,11	0,96 ± 0,10	-0,040	0,91 ± 0,16	0,95 ± 0,14	0,064
		Harakiri	0,00 ± 0,00	0,00 ± 0,00	0	0,00 ± 0,00	0,00 ± 0,00	0	0,00 ± 0,00	0,00 ± 0,00	0
	Bax/Bak like	Bax	3,06 ± 0,13	2,95 ± 0,13	-0,056	2,90 ± 0,85	2,06 ± 0,05	-0,492	2,12 ± 0,30	2,29 ± 0,52	0,115
		Bak	1,98 ± 0,11	2,07 ± 0,13	0,065	1,93 ± 0,55	1,50 ± 0,10	-0,358	1,24 ± 0,20	1,27 ± 0,26	0,029
		Bok	0,00 ± 0,00	0,00 ± 0,00	0	0,00 ± 0,00	0,00 ± 0,00	0	0,00 ± 0,00	0,00 ± 0,00	0
	Others	Bcl-G	0,00 ± 0,00	0,00 ± 0,00	0	0,00 ± 0,00	0,00 ± 0,00	0	0,00 ± 0,00	0,00 ± 0,00	0
		Bcl-Rambo	1,99 ± 0,09	1,89 ± 0,04	-0,075	1,58 ± 0,20	1,41 ± 0,14	-0,157	1,59 ± 0,23	1,69 ± 0,26	0,090
		MAP1	0,48 ± 0,05	0,36 ± 0,06	-0,444	0,49 ± 0,14	0,31 ± 0,04	-0,651	0,41 ± 0,06	0,30 ± 0,07	-0,448
		Boo/Diva	0,00 ± 0,00	0,00 ± 0,00	0	0,00 ± 0,00	0,00 ± 0,00	0	0,00 ± 0,00	0,00 ± 0,00	0
IAP Family		XIAP	0,20 ± 0,02	0,22 ± 0,02	0,146	0,30 ± 0,07	0,22 ± 0,03	-0,420	0,29 ± 0,09	0,20 ± 0,03	-0,563
		NIAP	0,00 ± 0,00	0,16 ± 0,01	0	0,98 ± 0,10	1,72 ± 0,13	0,819	2,24 ± 0,26	2,31 ± 0,44	0,042
		IAP1	6,04 ± 0,30	7,17 ± 0,13	0,246	4,91 ± 0,78	5,23 ± 0,60	0,089	6,06 ± 0,74	5,59 ± 1,06	-0,119
		IAP2	1,05 ± 0,04	1,32 ± 0,16	0,335	1,39 ± 0,14	1,64 ± 0,13	0,233	1,68 ± 0,23	3,01 ± 0,41	0,840
		LIVIN	0,00 ± 0,00	0,00 ± 0,00	0	0,00 ± 0,00	0,00 ± 0,00	0	0,00 ± 0,00	0,00 ± 0,00	0
		Apollon	13,34 ± 0,47	13,64 ± 0,56	0,032	11,15 ± 0,70	8,82 ± 0,80	-0,337	9,71 ± 1,65	8,48 ± 1,76	-0,196
Miscellaneous		P19	0,46 ± 0,02	0,65 ± 0,06	0,481	0,74 ± 0,20	0,93 ± 0,02	0,322	0,70 ± 0,08	0,77 ± 0,06	0,119
		CFLAR	6,76 ± 0,12	6,94 ± 0,39	0,036	5,32 ± 0,25	5,19 ± 0,38	-0,035	4,83 ± 0,47	4,56 ± 0,71	-0,082
		GzmB	0,00 ± 0,00	0,00 ± 0,00	0	0,22 ± 0,20	0,93 ± 0,16	2,065	0,26 ± 0,03	0,23 ± 0,04	-0,160
		Perforin1	2,91 ± 0,10	2,64 ± 0,30	-0,139	0,75 ± 0,21	1,18 ± 0,22	0,650	0,48 ± 0,08	0,26 ± 0,05	-0,911
		Apaf1	2,23 ± 0,15	2,46 ± 0,06	0,141	2,54 ± 0,25	3,95 ± 0,34	0,635	1,79 ± 0,34	2,39 ± 0,34	0,415
		Smac/Diablo	1,89 ± 0,20	1,50 ± 0,10	-0,332	1,23 ± 0,40	1,19 ± 0,09	-0,046	1,40 ± 0,10	1,11 ± 0,15	-0,333
		AIF	0,94 ± 0,05	0,93 ± 0,07	-0,020	0,96 ± 0,05	0,79 ± 0,05	-0,281	0,79 ± 0,16	0,67 ± 0,10	-0,248
		HtrA2	0,82 ± 0,16	0,82 ± 0,15	-0,002	0,85 ± 0,04	0,87 ± 0,05	0,022	0,95 ± 0,16	1,53 ± 0,25	0,681
		P21	0,00 ± 0,00	0,00 ± 0,00	0	0,00 ± 0,00	0,00 ± 0,00	0	0,00 ± 0,00	0,00 ± 0,00	0
Housekeeping		B2M	11,52 ± 0,14	11,37 ± 0,76	-0,018	12,89 ± 3,47	15,11 ± 5,99	0,229	15,11 ± 9,89	18,05 ± 12,05	0,256
		GUS	1,11 ± 0,02	1,04 ± 0,03	-0,093	1,08 ± 0,14	0,83 ± 0,13	-0,379	0,97 ± 0,21	0,77 ± 0,16	-0,343
		TBP	2,40 ± 0,13	2,05 ± 0,06	-0,225	1,49 ± 0,01	1,32 ± 0,05	-0,176	1,38 ± 0,15	1,04 ± 0,15	-0,410
		PAK2	10,33 ± 0,09	8,56 ± 0,14	-0,271	6,96 ± 1,15	5,92 ± 0,76	-0,233	7,19 ± 1,53	5,26 ± 0,92	-0,451

Summarizing discussion

7

The studies described in this thesis aimed to provide a better understanding of the brain's intrinsic immune suppressive systems with respect to a severe neuroinflammatory disease: multiple sclerosis (MS). Although the results are separately discussed in each chapter, I will here summarize the findings, highlight their importance as a whole, and give some suggestions for future research.

Immune suppressive systems in a balanced immune system

As Matsue in 2005 aptly expressed: 'the immune system must accomplish two opposing tasks: inducing efficient immunological protection against potentially harmful pathogens and, at the same time, preventing excessive and prolonged immunological responses that may lead to tissue destruction'.[248] In other words, proper functioning of the immune system requires a well regulated immunological balance maintained by both activating and inhibitory signals. Defects in either one of these signals consequently results in imbalanced immunity. Because the majority of studies regarding inflammatory diseases have focused on immune activators, the contribution of intrinsic immune suppressors has been underestimated, but is clearly equally important. Examples of potent immune suppressive systems required for immunological balance are cytotoxic T lymphocyte antigen (CTLA)-4 in T cells, and TREM2, CD47-SIRPα and CD200-CD200R in myeloid cells. For the latter pair of molecules, this concept was recently illustrated in the lung, using influenza as a model.[181] Alveolar macrophages are immune suppressed by high CD200R expression and during influenza infection CD200R levels further increased, which is suggested to diminish inflammation. Blocking CD200R signaling using $CD200^{-/-}$ mice resulted in increased viral clearance due to enhanced immune reaction, but coincided with increased mortality as inflammation was excessive. Triggering CD200R with agonistic reagents in turn dampened inflammation. This study demonstrates that the role of inhibitory mechanisms such as CD200-CD200R interaction relies in setting a threshold for immune activation and, when this is exceeded and inflammation has established, further provides a site-specific mechanism to control the immune response and prevent disproportionate tissue damage.

Physiological implications of immune suppressive systems

Immune regulation in the CNS

Special care for overzealous immune reactions should be taken in the CNS. Whereas most other organs are able to repair inflammatory damage, neurons hardly regenerate upon injury, and therefore need to be protected in several ways. The

extremely high expression of CD200 (and CD47) in the brain likely increases the threshold for inflammation and maintains an immune suppressed environment (**chapters 2** and **3**).[111,120] In MS patients however, myelin breakdown, axonal damage and neuronal loss ensue from seemingly uncontrolled inflammation. Apparently, the immunological balance is lost, which may not only be due to enhanced immune activating factors, but may also be ascribed to decreased immune inhibitory input through CD200 and CD47 as shown in **chapter 2**.[111] The consequence is a diminished threshold which facilitates immune activation, and may thus underlie uncontrolled inflammation. However, it seems that the brain has additional mechanisms to restore homeostasis during pathology, by enhancing CD200-CD200R interaction, by induction of CD200 expression on astrocytes in MS lesions (**chapter 3**). This might be similar to the situation studied in the lung.[181] Nevertheless, astrocytes cannot sufficiently compensate the loss of CD200 as overall expression of CD200 in MS lesions is decreased, which might be due to loss of expression of this molecule on neurons and oligodendrocytes, as these are the two main cell types expressing CD200 and are most heavily affected by the disease.

CD200-CD200R interaction is not the only system with immune modulatory functions in the brain. Other examples are glucocorticoids (GCs) produced from hypothalamo-pituitary-adrenal (HPA)-axis activation and sex hormones as estrogen and testosterone. Both types of hormones have multiple actions including anti-inflammatory and neuroprotective effects.[249-251] Upon treatment, glucocorticoids and estrogens are potentially beneficial for MS patients as they can reduce the volume and number of gadolinium enhancing lesions.[46,152] In many MS patients, endogenous cortisol production is increased although clinical effectiveness is yet to be determined.[252-254] This indeed may form a mechanism to control the immune system, as demonstrated by the finding that suppression of the HPA-axis by active hypothalamic MS lesions correlated with a worsened disease course.[255] Protective effects of endogenous estrogen are suggested by studies showing that elevated levels of estrogen during the third trimester of pregnancy are associated with a decrease in MS symptoms.[256] Its immune regulatory capacity is demonstrated by the observation that symptoms rapidly exacerbate post-partum, when estrogen levels drop.[256] The expression analysis of several genes in MS lesions, described in **chapter 2** of this thesis, shows amongst others an increased amount of ERα in MS lesions, possibly reflecting an endogenous mechanism to modulate local inflammation. Interestingly, in neurons of the human paraventricular nucleus, ERα colocalizes with CRH, the driving force of the HPA-axis.[257] Since estrogen response elements are present in the promoter region of CRH,[258] one of the mechanisms by which estrogen exerts its beneficial effects may be the activation of the HPA-axis

and thus the enhancement of glucocorticoid levels.

The systems mentioned above are hypothesized to act in response to inflammation in order to restore the immunological balance by inducing CD200 expression in astrocytes, activating the HPA-axis to release cortisol and to increase the expression of estrogen receptors. Interestingly, inflammation in MS lesions at some point resolves, but the mechanisms behind this ceased activity are currently unknown. It might be speculated that the systems mentioned above and described in this thesis are involved in halting inflammation in an established lesion. In contrast to fluctuating hormone levels, CD200 and CD200R are constitutively expressed in the brain. The sustained neuron-glia and glia-glia interactions (**chapter 3**) would thus incessantly control the immune response, suggesting that this system may therefore be more effective in continuously balancing immunity compared to systems that are induced upon inflammation. The decreased expression of CD200 would therefore significantly contribute to the imbalanced immunity in MS. Accordingly, these systems are excellent targets for therapy, as will be discussed below.

Shaping of immune responses by CD200-CD200R

CD200-CD200R interaction may not only be involved in suppression of the innate immune system, but potentially also in modeling its response. The data presented in **chapter 4** shows that IL-4 induced alternative activation of macrophages (M2 cells) triggers enhanced CD200R expression on these cells. M2 cells display an anti-inflammatory phenotype and are known to mediate tissue remodeling and repair as well as to induce Th2 responses by expressing anti-inflammatory cytokines.[22,23] As the effects of CD200R triggering, i.e. decreased production of IL-6 and TNF,[124] are in line with those seen in M2 cells, it is likely that CD200R signaling is responsible for many M2 cell activities. In MS, infiltrating and activated macrophages have a pro-inflammatory phenotype, but macrophages that have ingested myelin switch to an anti-inflammatory phenotype consistent with M2 cells.[29] In concordance, **chapter 3** of this thesis shows that foamy macrophages express CD200R. Notably, this is after myelin ingestion, whereas an early shift to an anti-inflammatory phenotype would possibly prevent inflammation and lesion development. Several current therapies that appear beneficial in MS treatment can shift a type I to a type II immune response (**chapter 1**). Proper activation of CD200R by a therapeutic agonist could therefore be highly beneficial in MS, not only to suppress immune activity, but also to shift towards a type II immune response with a more favorable outcome.

To achieve a switch in the immune response in MS lesions, CD200R signaling in the CNS should be increased, for example by enhancing its ligand, CD200. However, the regulation of CD200 is still obscure. Although a single group claimed that

a remarkably high dose of IL-4 enhances neuronal CD200 expression,[193,259] the experiments with physiological levels of IL-4, described in **chapter 5** of this thesis, did not confirm this. Indeed, IL-4 may play a role in the regulation of macrophage activation, but rather by inducing CD200R expression on polarized macrophages as discussed above (**chapter 4**). In **chapter 4** and **5**, we show that CD200R is also expressed on microglia. Interestingly, CD200R expression on these cells is not enhanced by IL-4, indicating that microglia either do not polarize, have already polarized, or are influenced by other factors than IL-4. Nevertheless, regarding MS it is important to note that the expression of CD200R in lesions was unchanged,[111] indicating that it is available as therapeutic target. In absence of knowledge on how to enhance endogenous CD200 expression, administration of soluble CD200 or a CD200R agonistic antibody may provide a good therapeutic approach to diminish and shape the inflammatory response and regain homeostasis in the brain.

Peripheral immune regulation by GITR-GITRL

Apart from studies concerning immune suppression in the brain, we also studied a mechanism of peripheral immune homeostasis. **Chapter 6** shows that interaction of glucocorticoid-induced tumor necrosis factor receptor family-related protein (GITR) and its ligand (GITRL) is involved in balancing the adaptive immune system by increasing both regulatory and effector T cell populations. Functional consequences of controlling the numbers of both regulatory and effector T cells by GITR stimulation appeared protective in experimental autoimmune encephalomyelitis (EAE), as it significantly delayed disease onset. In MS patients, regulatory T cells were thought to have reduced suppressive activities,[260,261] but this was recently revised when non-regulatory $CD127^{hi}$ expressing T cells were removed from the population of $CD4^{+}CD25^{hi}$ regulatory T cells that subsequently had similar suppressive capacities when compared to healthy individuals.[262] Regulatory T cells are highly important in controlling potentially harmful responses for example to self-antigens.[263] Although regulatory T cell function apparently is not altered in MS, the $CD127^{hi}$ T cell population was highly activated in patients. These results indicate that the imbalance in immune regulation is also displayed in the T cell compartment. Hence, GITR-GITRL signaling could be an interesting mechanism to rebalance responses of the peripheral adaptive immune system.

Clinical implications of immune suppressive systems in the CNS

As mentioned above, immune suppressive systems are essential for a proper functioning and balanced immune system. Especially in the CNS where they provide

in concert with other mechanisms such as the blood-brain barrier a constitutive immune suppressed environment. In this way the immunological survey in the CNS, predominantly carried out by microglia, is still occurring, but will not elicit substantial inflammatory responses. This is highly important, because protection of neurons and other CNS cell types from inflammation-mediated degeneration is paramount, as neuronal damage will hardly be repaired. In several conditions however, strong microglia activation is observed. MS is an extreme example, where activated microglia, but also infiltrating macrophages are thought to be effector cells driving disease development. Microglia activation is also evident in stroke, brain trauma, Alzheimer's disease, Parkinson's disease and in ageing, where they are considered to contribute to neurodegeneration.[264-268] Evidence is growing on the relation of microglia activation with CD200 levels in these conditions. During ageing in rats, microglia activation showed a strong correlation with reduced amounts of CD200.[269] In humans, decreased immune suppression via CD200-CD200R has first been implicated in MS (this thesis), and recently a study in brains of patients with Alzheimer's disease elegantly showed a significant reduction in mRNA and protein expression levels of CD200 but also CD200R in the hippocampus of these patients.[132] Although direct evidence is still lacking, CD200 expression is furthermore suggested to be linked to microglia activation in Parkinson disease.[270] Enhancing CD200-CD200R interaction could therefore be beneficial in many neurodegenerative conditions where activated microglia may cause or enhance neuropathology.

A prerequisite for a potential therapeutic target such as the CD200-CD200R interaction is its sufficient capacity to effectively dampen immune activation. The spontaneous activation of microglia in absence of CD200 already indicates the power of this immune suppressive system, but its strength has been further demonstrated in studies concerning tumor immunology and anti-viral responses. For example, CD200 expression is enhanced on several tumors and is subsequently associated with a decreased anti-tumor immune response and worsened survival prognosis.[131,271-273] Also several viruses express CD200-like molecules that have been shown to bind to CD200R and indeed are able to suppress myeloid immune responses.[274-277] Although elevated levels of CD200 in these examples are undesired, they clearly demonstrate the effectiveness of CD200-CD200R interaction in dampening immune responses. *Wld*^{S} mice have inherently elevated levels of CD200 in the CNS.[129] Importantly for MS, this enhanced CD200 expression appeared responsible for suppression of inflammation and protection against EAE compared to wild type littermates. Conclusively, these studies indicate that CD200-CD200R interaction can be expected a powerful tool for treatment of MS.

To enhance immune suppression as MS therapeutic, it is important that the drug is able to enter the brain, where the imbalance in the immune system is most evident. Whereas most compounds cannot cross the blood-brain barrier and thus cannot enter the brain in healthy individuals, this might be relatively easy in MS, since damage of the blood-brain barrier is a well known feature in active MS lesions. Hence, systemically administered small molecule CD200R agonists like CD200 fusion proteins or agonistic CD200R antibodies, likely enter the brain easily and could thus act locally to suppress inflammation and restore the immune suppressed environment. Maximizing the stability and increasing the affinity of CD200R agonists would furthermore be important in the development of such a therapeutic.

Future directions

Many questions still need to be answered and necessitate further research focusing on immune suppressive systems in MS. As discussed before, CD200 expression can be induced in astrocytes in MS. This indicates that CD200 expression is regulated, but it remains unclear what factor is responsible. Similarly, the intensity of CD200 expression varies among different tissues, with the highest expression found in the CNS.[120] So far, the influence of cytokines on CD200 has been studied, but other soluble or membrane-bound proteins should also be studied. For example, we showed that cytokines could not affect CD200 expression in cultured neuroblastoma cells, but these cells seemed to express higher levels of CD200 protein when clusters were formed, as seen by immunohistochemistry (data not shown). Although this could not yet be confirmed quantitatively on mRNA or protein level, this observation argues for involvement of cell adhesion molecules. In addition, the role of different CNS cell types in influencing CD200 expression could also be studied.

An important question is what the cause is for the decreased amount of CD200 and CD47 in MS lesions. It could be a simple reflection of damaged neurons and oligodendrocytes. However, CD47 was also decreased in white matter directly adjacent to MS lesions (**chapter 2**) and recent preliminary data suggest a reduction in expression of both CD200 and CD47 in normal appearing white matter that does not directly surround MS lesions as tissue without MS lesions was used and gene expression data were consistent within this tissue as well as in the different donors (J. Melief, pers. comm.). Since activation of microglia, as demonstrated by up-regulation of MHC class II molecules, is not yet detectable in these areas, these findings corroborate the concept that immune suppression in the brains of MS patients is impaired, leading to a decreased immunological threshold that hence facilitates

the development of inflammatory foci. Indeed, this may be indicative of an event prior to neuronal and oligodendrocyte injury, but does not exclude the possibility that affected neurons or oligodendrocytes are an initial key factor in MS lesion ontogeny since decreased expression of neurofilament and myelin basic protein (MBP) can also be found in white matter not directly surrounding MS lesions (J. Melief). Future research should therefore focus on the mechanisms of the reduced expression of the immune suppressive molecules in the CNS of MS patients and whether this correlates to microglia activation, which is linked to the earliest stages of lesion development,[6,33,34] in order to understand the cause and consequence of this hampered immune balance.

In the gene encoding CD200, 196 single nucleotide polymorphisms (SNPs) have been identified, of which 7 result in an amino acid change (www.genecards.org). In the CD200R gene, 199 SNPs are known including 7 changes in amino acid sequence. However, it is at present unknown whether these alterations have functional consequences. SNP analysis should therefore be performed on post-mortem brain tissue in order to study the associations and implications of these polymorphisms with MS or any other neurodegenerative disease.

CD200 in the brain most obviously binds CD200R on perivascular macrophages and microglia. The biology of human microglia in MS is a largely unexplored field, with the most important reason that these cells are hardly accessible and methods to isolate them from post mortem tissue in a pure form are currently limited. Based on previous work on microglia isolations from rodent brains described by Sedgwick and colleagues,[162,184,185] **chapter 5** of this thesis shows a reliable and rapid method of isolating and culturing resident microglia from human post-mortem tissue. This opens the door to study the dynamics of these cells from control subjects and patients with different neurodegenerative diseases, including MS. Since macrophage activation is known to be diverse, depending on the stimulus, it would be highly interesting to know whether microglia also show this diversity upon activation and if this would correlate with neurotoxic or neuroprotective effects that have been ascribed to activated microglia.[31] To obtain information on the activation and polarization status of human macrophages and microglia, one could perform a differential screening of gene and protein expression for example directly after isolation of the cells or after stimulation with pro- and anti-inflammatory compounds. In addition, cells can also be compared for example between relatively young donors and older donors, or between donors with no disease of the central nervous system and MS patients. Especially in older donors and in MS patients, microglia are expected to show a higher activation status compared to microglia derived from younger or control donors. These screenings can also be

performed on macrophages and microglia derived from mice as the higher availability of tissues compared to human donors is an important advantage. However, macrophage polarization in mice may have different underlying mechanisms compared to those in human (**chapter 4**), indicating that data from mice macrophages and microglia can not unquestioningly be extrapolated to humans. Finally, microglia and macrophages that have ingested myelin in MS lesions, display an alternatively activated phenotype[29] and express CD200R (**chapter 3**). In following studies, it should be tested whether inducing high CD200R expression on non-myelin laden macrophages prevents inflammation-mediated phagocytosis such as seen in MS. In line with this, it should also be studied if CD200 could mediate polarization of macrophages, once classically activated, towards alternative activation and how this affects phagocytosis and inflammation.

Additional mouse studies could provide important information on the role of macrophage regulation in the disease model for MS. For instance, depleting macrophages from CD200$^{-/-}$ mice would identify the role of peripheral macrophages in EAE in the absence of CD200. Conditional knockouts of CD200R on macrophages, by using the Lysozyme M(Cre)[280] or F4/80(Cre) mice, is expected to increase the development and severity of EAE because of a lack of inhibition of these cells. In addition, bone marrow chimaeras of WT and CD200R$^{-/-}$ mice are considered valuable in dissecting the role of blood-derived macrophages versus resident microglia.

Other mechanisms that control the activation of microglia, such as CD47-SIRPα interaction should be further studied as well. For example, their broad expression pattern is comparable to that of CD200, but the cell types positive for these molecules have not been identified in detail, neither are factors that can regulate their expression. CD47-SIRPα interactions play a role in phagocytosis of erythrocytes, but it is currently unknown whether it affects myelin phagocytosis. The effects of CD47-SIRPα and of CD200-CD200R on phagocytic capacities of macrophages and microglia should be elucidated in future studies. Unfortunately, double knockout mice for CD200 and CD47 will not be helpful as the mice appear not viable (R.M. Hoek, pers. comm.). TREM2 which in the brain is mainly expressed by microglia, has been demonstrated to have immune suppressive activities in EAE.[278,279] However, its ligand is still unknown and limited information is available on the mechanisms by which TREM2 exerts its anti-inflammatory functions. More research is therefore needed to elucidate the immune suppressive properties of TREM2.

Finally, the most important and relevant question to be answered is whether CD200-CD200R interaction alone, or in combination with the other intrinsic immune suppressive mechanisms mentioned above, can indeed restore balanced immunity in MS and can finally stop the progression of this very disabling disease.

Samenvatting

8

Multiple sclerose (MS) is een ernstige ziekte van het centrale zenuwstelsel waarbij het immuunsysteem de beschermlaag van de zenuwen, het myeline, aanvalt en beschadigt. Zulke beschadigde plekken in de hersenen worden lesies genoemd en leiden ertoe dat zenuwsignalen niet goed meer worden overgebracht en daardoor vallen verschillende lichaamsfuncties uit. Eenmaal beschadigd kunnen zenuwcellen nauwelijks herstellen. Omdat de oorzaak van MS nog niet bekend is, zijn huidige therapieën erop gericht om zoveel mogelijk schade te voorkomen. Huidige therapieën kunnen tot op zekere hoogte klachten verminderen, maar kunnen MS nog niet stoppen. Er moet dus verder gezocht worden naar andere mechanismen die effectiever zijn in de behandeling van MS.

Eén van de belangrijkste gebeurtenissen in MS die leidt tot het ontstaan van lesies, is de activatie van bepaalde type immuuncellen, namelijk de macrofaag (betekent: grote eter), en microglia (kleine macrofaag-achtige cellen die alleen in de hersenen voorkomen). Normaal gesproken zijn deze cellen verantwoordelijk voor het opruimen van bacteriën en afvalstoffen omdat ze deze fagocyteren, ofwel letterlijk opeten. In MS fagocyteren ze echter myeline. Waarom deze cellen in MS zo actief worden is niet bekend. Wel kennen we verschillende mechanismen die deze cellen kunnen activeren, of juist kunnen remmen. De juiste balans moet ervoor zorgen dat cellen alleen actief worden wanneer dit nodig is, zoals bij het opruimen van bacteriën. In MS lijkt de balans verstoord, want macrofagen en microglia zijn actief, terwijl ze dat niet horen te zijn.

Dit proefschrift bevat verschillende studies naar een specifiek mechanisme om macrofagen en microglia af te remmen. Dit mechanisme berust op de interactie tussen twee eiwitten: CD200 en de bijbehorende receptor CD200R. CD200 komt veel voor op hersencellen maar CD200R zit alleen op immuuncellen, en met name op macrofagen en microglia. Als CD200 aan CD200R bindt, communiceren hersencellen dus met immuuncellen. Het signaal dat daarbij wordt doorgegeven kan de activatie van macrofagen en microglia remmen. Omdat in MS deze cellen actief zijn terwijl ze dat niet horen te zijn, hebben we MS lesies vergeleken met controle hersenweefsel om te bestuderen of CD200, CD200R en andere moleculen verstoord zijn in MS. Uit deze studie bleek inderdaad dat CD200, maar niet CD200R, verminderd is in MS lesies (beschreven in **hoodstuk 2**). We concluderen dat een verminderd remmend signaal een verstoorde balans kan opleveren waardoor de cellen makkelijker actief raken en ontstekingen in MS kunnen veroorzaken. Om meer te weten te komen over hoe precies CD200-CD200R interactie plaats kan vinden, hebben we vervolgens gekeken op welke cellen in de hersenen van controle donoren en MS patiënten CD200 en CD200R zit (**hoofdstuk 3**). Zenuwcellen en hun uitlopers brengen CD200 tot expressie, maar ook oligodendrocyten, de cel-

len die de myelinelaag maken. Astrocyten (cellen met een ondersteunde functie voor zenuwcellen en verantwoordelijk voor het vormen van littekenweefsel in MS lesies) maken geen CD200. Een uizondering hierop zijn astrocyten in en rondom MS lesies, die wel CD200 aanmaken. Deze verrassende bevinding wijst erop dat de hersenen een compensatie mechanisme hebben om opnieuw te proberen de geactiveerde immuuncellen te onderdrukken, alhoewel dit in MS niet goed lijkt te lukken omdat het de ziekte niet tegenhoudt.

Als bekend zou zijn welke stoffen zorgen voor de expressie van CD200 in de hersenen, zouden we deze kennis toe kunnen passen om het tekort in MS lesies op te heffen. Omdat zulke stoffen nog niet bekend zijn hebben we verschillende factoren onderzocht op gekweekte cellen uit het brein (**hoofdstuk 4** en **5**). Ondanks dat we meerdere stoffen onderzocht hebben, kunnen we tot nu toe nog geen enkele factor vinden die de hoeveelheid CD200 op gekweekte zenuwcellen kan verminderen of vermeerderen. Op gekweekte macrofagen echter, bleek CD200R met name toe te nemen door het molecuul interleukine-4 (IL-4). Dit is interessant omdat onder invloed van IL-4, macrofagen een alternatief activatie programma aanzetten, waardoor ze wel actief zijn, maar speciale ontstekingsremmende effecten hebben. Omdat CD200R specifiek op deze cellen lijkt te zitten, lijkt het erop dat CD200R niet zozeer de cel inactiveert, zoals altijd werd aangenomen, maar eerder bijdraagt aan deze ontstekingsremmende effecten. Microglia hebben ook CD200R, dus wilden we weten of de hoeveelheid ervan ook toe kan nemen na IL-4. Er is in het verleden weinig onderzoek naar microglia gedaan, omdat deze cellen moeilijk te isoleren zijn uit de hersenen. Daarom hebben we een speciale en unieke techniek ontwikkeld om onmiddelijk na obductie, uit humane hersenen microglia te isoleren en te kweken (**hoofdstuk 5**). Tot onze verrassing zorgt IL-4 niet voor een toename van CD200R op microglia. Hieruit concluderen we dat microglia, die ook de 'macrofagen van de hersenen' worden genoemd, niet identiek zijn aan andere weefsel-macrofagen en hun eigen, unieke capaciteiten hebben. Omdat onze techniek om microglia te isoleren goed werkt, is in de toekomst veel meer onderzoek naar deze cellen mogelijk.

In **hoofdstuk 6** laten we zien dat de interactie tussen CD200 en CD200R niet het enige mechanisme is om de juiste balans binnen het immuunsysteem te bewaren. Een ander mechanisme dat we onderzocht hebben is de interactie tussen de twee eiwitten GITR en GITRL in muizen die door een genetische aanpassing meer GITRL hebben dan normaal. Uit deze studie blijkt dat door middel van deze eiwitten, het aantal effector T cellen stijgt, maar ook het aantal regulatoire T cellen. Effector T cellen zijn immuuncellen die effectief een ontstekingsreactie veroorzaken en daardoor een infectie kunnen bestrijden. Regulatoire T cellen zijn immuuncellen die de

effector T cellen kunnen onderdrukken, zodat de ontsteking niet uit de hand loopt. In EAE, het diermodel voor MS, wordt duidelijk dat GITR-GITRL interactie beschermend werkt omdat het ontstaan van de ziekte wordt geremd. GITR-GITRL speelt dus een rol in de balans tussen deze twee T cel groepen in immuunreacties.

Dit proefschrift laat zien dat de interactie tussen CD200 en CD200R belangrijk is om het immuunsysteem in de hersenen in balans te houden. Nu dit in MS verstoord blijkt, zou CD200R wellicht een aangrijpingspunt kunnen zijn voor behandeling van MS. CD200 in de hersenen van MS patiënten was verlaagd, dus het geven van CD200 als medicatie zou het tekort op kunnen heffen en zou CD200R opnieuw voldoende kunnen stimuleren. Dit zal bovendien macrofagen en microglia zelf niet alleen afremmen, maar zou deze cellen ook nog kunnen aanzetten tot algehele ontstekingsremmende mechanismen zoals o.a. weefselherstel. Toekomstige studies zullen moeten uitwijzen wat de mogelijkheden zijn van CD200R stimulatie als toekomstige therapie voor MS.

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10

N. Koning, D.F. Swaab, R.M. Hoek, I. Huitinga
Distribution of the immune inhibitory molecules CD200 and CD200R in the control central nervous system and multiple sclerosis lesions suggests neuron-glia and glia-glia interactions.
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EMR1, the human homologue of F4/80, is an eosinophil specific receptor.
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R.W. van Olffen, **N. Koning**, K.P.J.M. van Gisbergen, F. Wensveen, E. Eldering, R.M. Hoek, J. Hamann, R.A.W. van Lier, M.A. Nolte
GITR triggering induces expansion of both effector and regulatory
$CD4^+$ T cells *in vivo*.
Submitted

N. Koning, M.C. van Eijk, W. Pouwels, M.S.M. Brouwer, G. Raes, I. Huitinga, R.M. Hoek, J. Hamann
Expression of the inhibitory CD200 receptor is associated with alternative activation of macrophages.
Submitted

N. Koning, D.F. Swaab, I. Huitinga, R.M. Hoek
Restoring immune suppression in multiple sclerosis.
Submitted

About the author

11

Nathalie Koning was born on January 18, 1981 in Tegelen, The Netherlands. In 1999 she received her secondary school degree at Chr. Gymnasium Sorghvliet in The Hague. The same year she started to study Biomedical Sciences at Leiden University. During this study, she performed a 5-month internship at the department of Pulmonary Diseases at the Leiden University Medical Center, supervised by dr. E.L. van Rensen and Prof. P.J. Sterk. An internschip of 9 months was performed at the department of Medical Pharmacology of the Leiden Amsterdam Center for Drug Research, supervised by dr. L. Enthoven, dr. M.S. Oitzl and Prof. E.R. de Kloet, where she studied the development of the hypothalamic-pituitary-adrenal (stress)- axis in newborn mouse infants subjected to repeated maternal deprivation. In 2004, she graduated *cum laude* and started as a PhD-student on a neuro-immune project at the Netherlands Institute for Neuroscience and at the department of Experimental Immunology in the Amsterdam Medical Center in Amsterdam. Her research focused on immune suppressive mechanisms to control macrophages and microglia in multiple sclerosis. The studies, described in this thesis, were funded by the Dutch MS Research Foundation, and were performed under supervision of dr. I. Huitinga and dr. R.M. Hoek, and Prof. D.F. Swaab and Prof. R.A.W. van Lier. Since October 2008, Nathalie Koning is positioned as a post-doc in the group of Prof. Y. van Kooyk, at the department of Molecular Cell Biology and Immunology at the VU Medical Center in Amsterdam, where her work is focused on the immune modulatory effects that glycans present on milk proteins can have on dendritic cells.

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12

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